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Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

Application Data Sh et

Application Information

Application number::	Not Yet Assigned
Application Type::	Provisional
Subject Matter::	Utility
Suggested Group Art Unit::	Not Yet Assigned
CD-ROM or CD-R?::	None
Sequence submission?::	No
Computer Readable Form (CRF)?::	No
Title::	METHOD OF PREPARATION OF RNA CAPABLE OF TARGET-DEPENDENT CIRCULARIZATION AND TOPOLOGICAL LINKAGE
Attorney Docket Number::	367593000500
Request for Early Publication?::	No
Request for Non-Publication?::	No
Number of Drawings::	19
Small Entity?::	Yes
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

Applicant Information

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Correspondence Customer Number::	25226

Representative Information

Representative Customer Number::	25226

Application Data Sheet

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Open and Cl sed Complexes between RNA Lass s and Targets

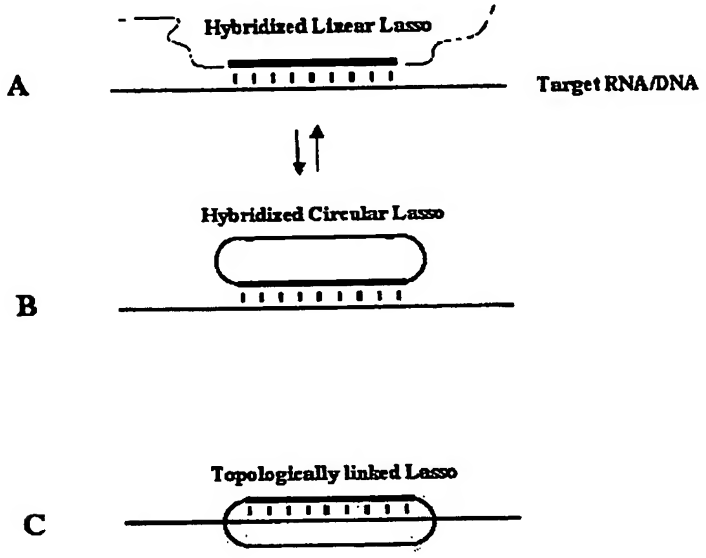


Figure 1

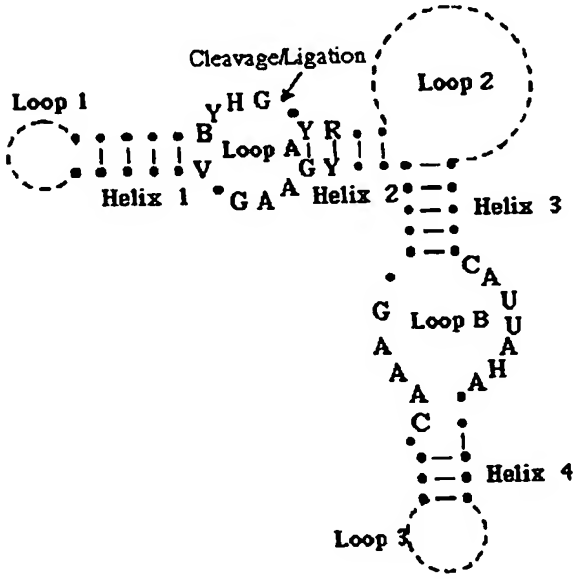
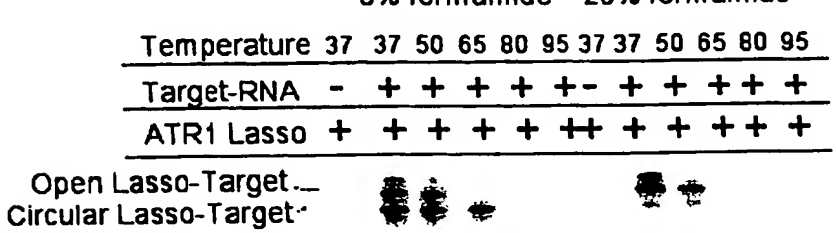
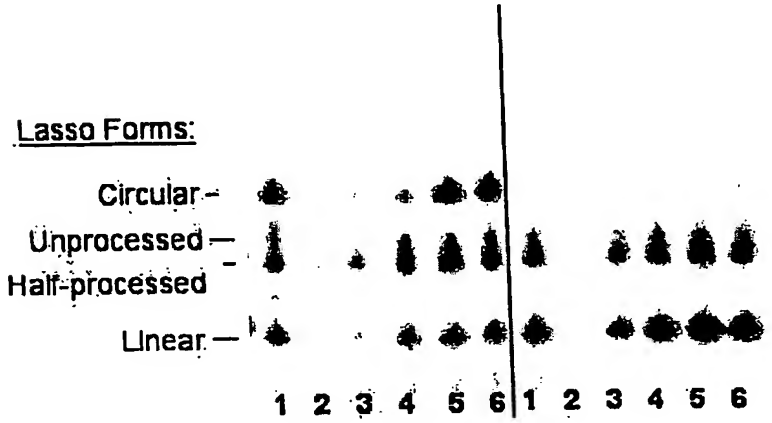

Cleavage/Ligation
Loop 1
Loop 2
Loop A
Loop B
Helix 1
Helix 2
Helix 3
Helix 4
Loop 3

Figure 2

Circulariz d Lassos form str nger compl xes with target RNA than linear Lassos





Complexes were formed at 37°C for 2 hrs. Buffer: 10mM $MgCl_2$, 50 mM Tris-HCl(pH 8) with or without formamide as indicated
Displacement ProcedureComplexes with one volume of loading buffer added have been incubated 2 min at temperature indicated followed by ice-cooling before loading in the gel (PAGE: 6% PAGE/8M UREA)

•Open and closed lasso-target complexes have different mobility shifts
•Circular lasso-target complexes are more stable than complexes with open lassos

Figure 3

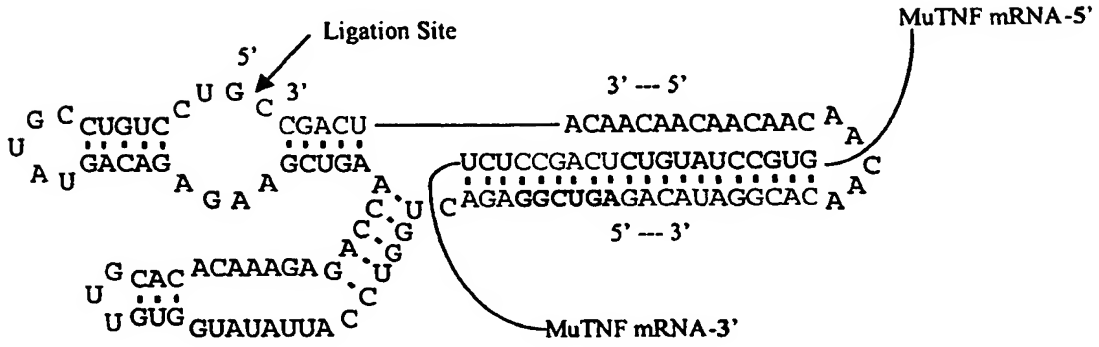
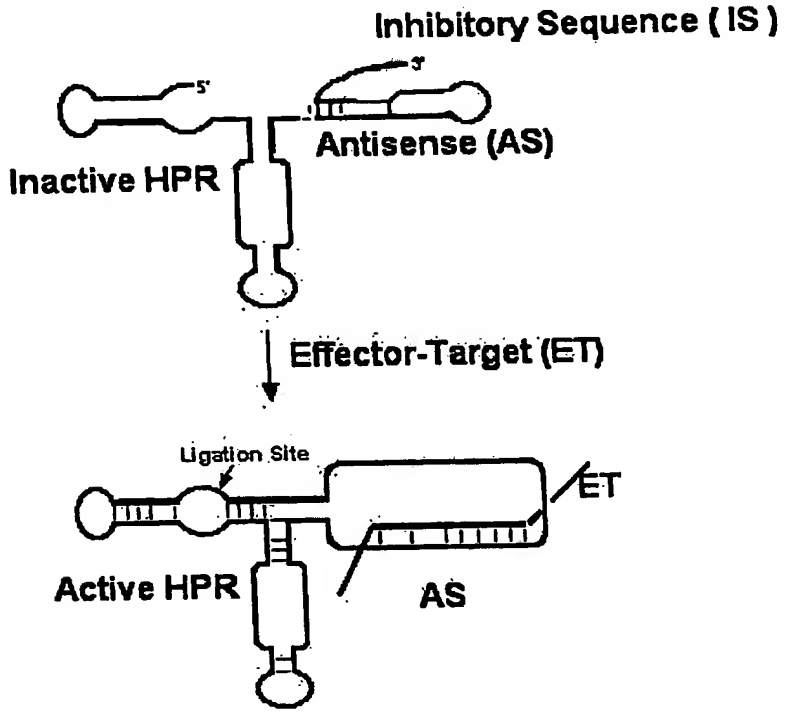

Ligation Site
MuTNF mRNA-5'
5'
3'
3' --- 5'
CUGUC
CGACU
ACAACAACAACAAC
GACAG
GCUGA
UCUCCGACUCUGUAUCCGUG
AGAGGCUGAGACAUAGGCAC
5' --- 3'
CAC
ACAAAGA
GUG
GUAUAUUA
MuTNF mRNA-3'

Figure 4

Goal: Target-Mediated Allosteric Regulation of Lasso HPR for more efficient circularization around target

Allosteric regulation of a Lasso hairpin ribozyme can be achieved by introducing inhibitory internal base pairing sequences that bind to a Lasso terminal sequence, preventing self-processing and circularization of the Lasso prior to hybridization with the target RNA.



Some advantages of allosteric regulation:
- Increased binding efficiency to target RNA
- Improved yield of topologically linked lasso-target complexes
- Increased specificity of lasso-target interaction

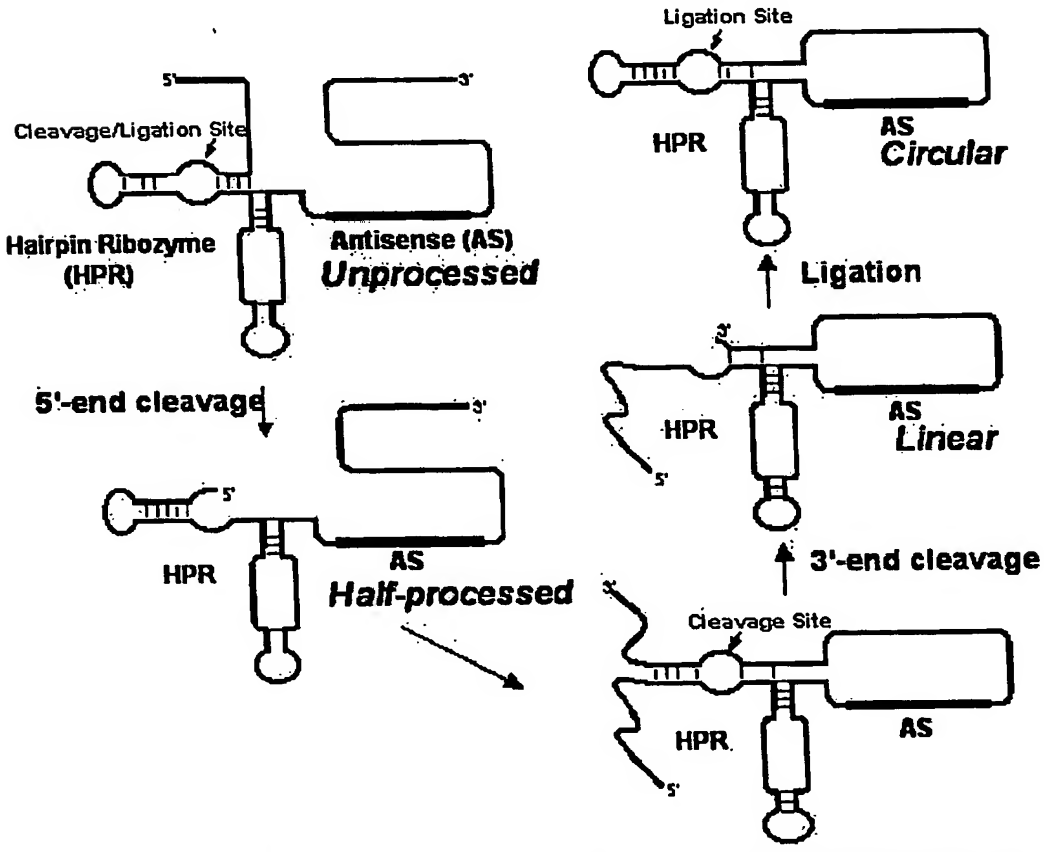
Figure 5

RNA Lassos

- catalytic RNA molecules that are designed to hybridize to target RNAs and ligate around the target RNA, creating a topologically linked complex

- contain a catalytic hairpin ribozyme (HPR) domain and a target hybridization domain

HPR Lasso Self-Processing

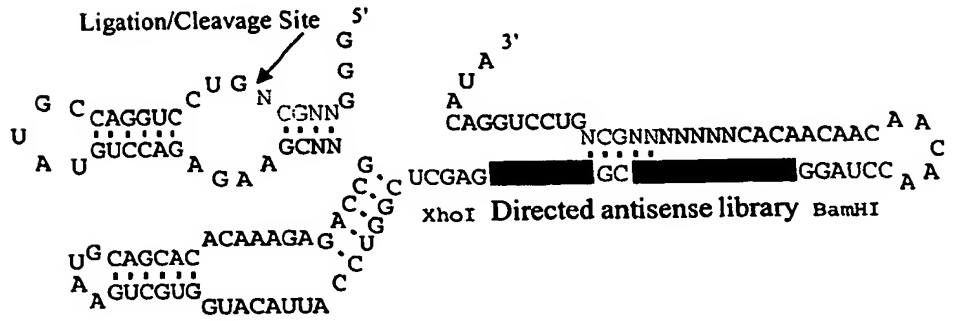


Lassos can exist in both "linear" and "circular/ ligated" forms

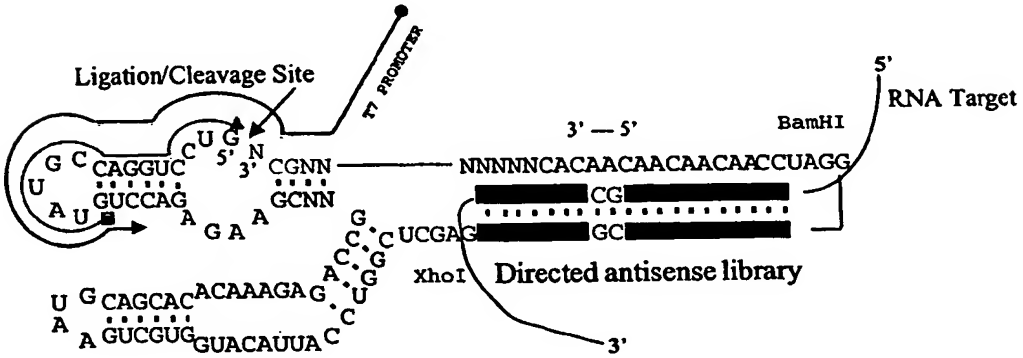
Figure 6

c

a



b



c

Detection and Amplification of Circularizing Lassos by RCA-PCR

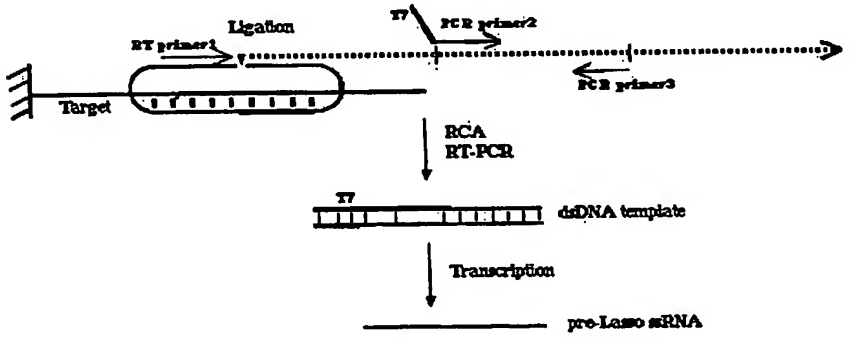


Figure 7

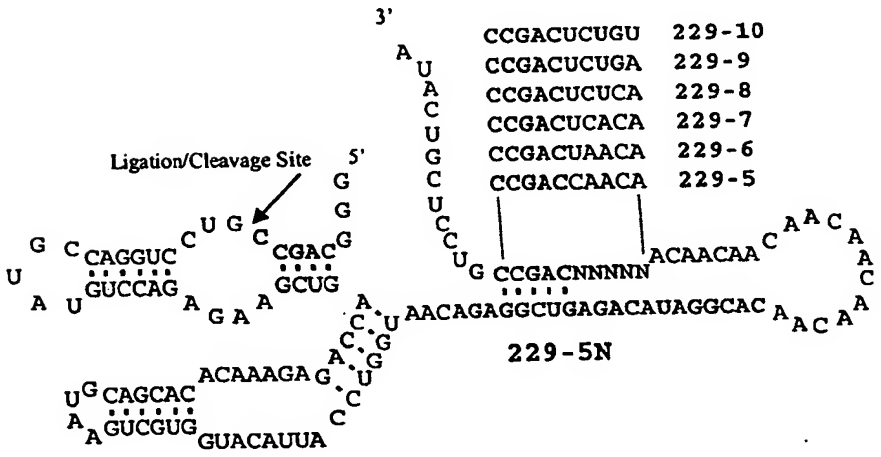

3'
CCGACUCUGU 229-10
CCGACUCUGA 229-9
CCGACUCUCA 229-8
CCGACUCACA 229-7
CCGACUAACA 229-6
CCGACCAACA 229-5
Ligation/Cleavage Site
5'
CCGACNNNNN
ACAACAA
AACAGAGGCUGAGACAUAGGCAC
229-5N
CAGGUC
GUCCAG
CGAC
GCUG
ACAAAGA
CAGCAC
GUCGUG
GUACAUUA

Figure 8

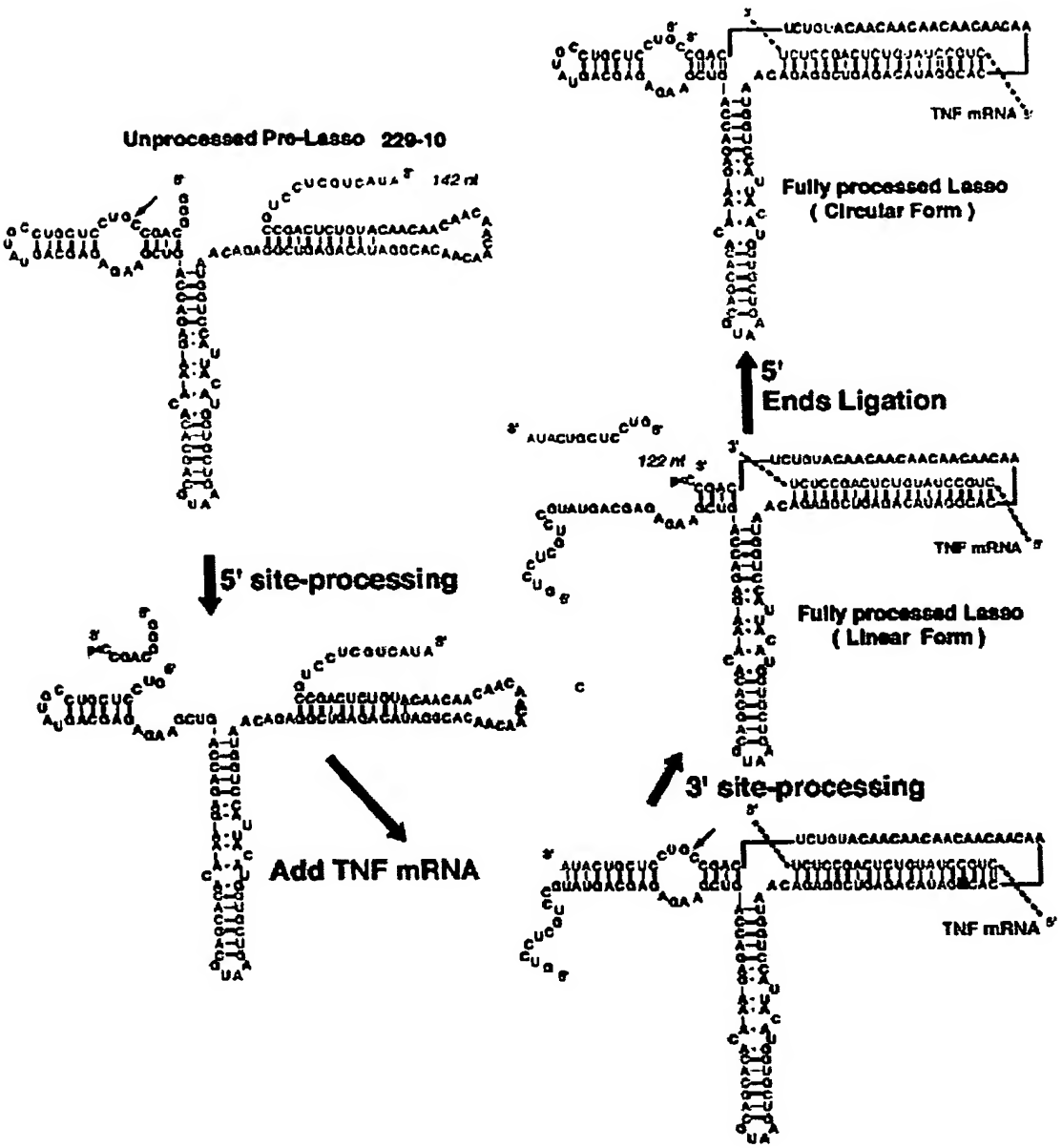

Unprocessed Pre-Lasso 229-10
142 nt
5' site-processing
Add TNF mRNA
3' site-processing
5' Ends Ligation
Fully processed Lasso (Circular Form)
Fully processed Lasso (Linear Form)
122 nt
TNF mRNA

Figure 9

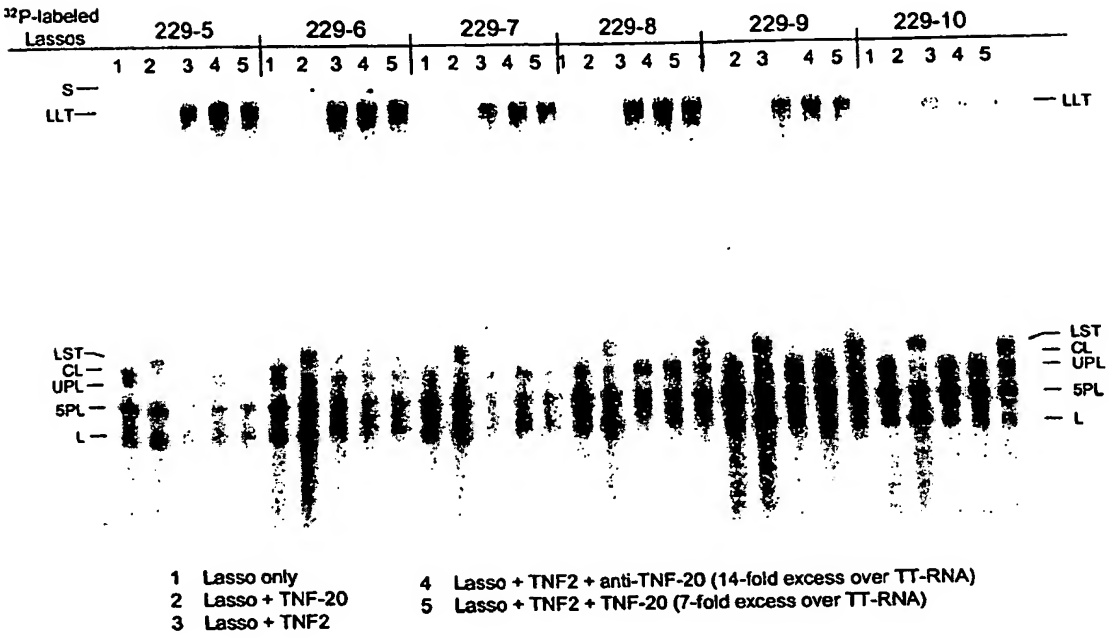

32P-labeled Lassos
229-5
229-6
229-7
229-8
229-9
229-10
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
S
LLT
LLT
LST
CL
UPL
5PL
L
LST
CL
UPL
5PL
L
1 Lasso only
2 Lasso + TNF-20
3 Lasso + TNF2
4 Lasso + TNF2 + anti-TNF-20 (14-fold excess over TT-RNA)
5 Lasso + TNF2 + TNF-20 (7-fold excess over TT-RNA)

Figure 10

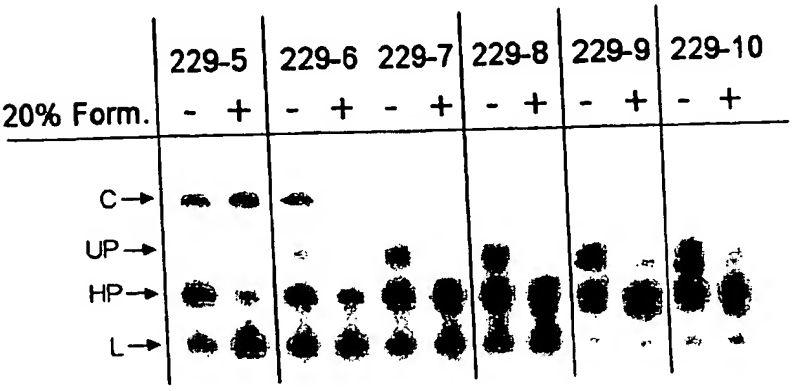


Figure 11

Lasso	229-5		229-6		229-7		229-8		229-9		229-10	
TT-RNA	-	+	-	+	-	+	-	+	-	+	-	+

Figure 12

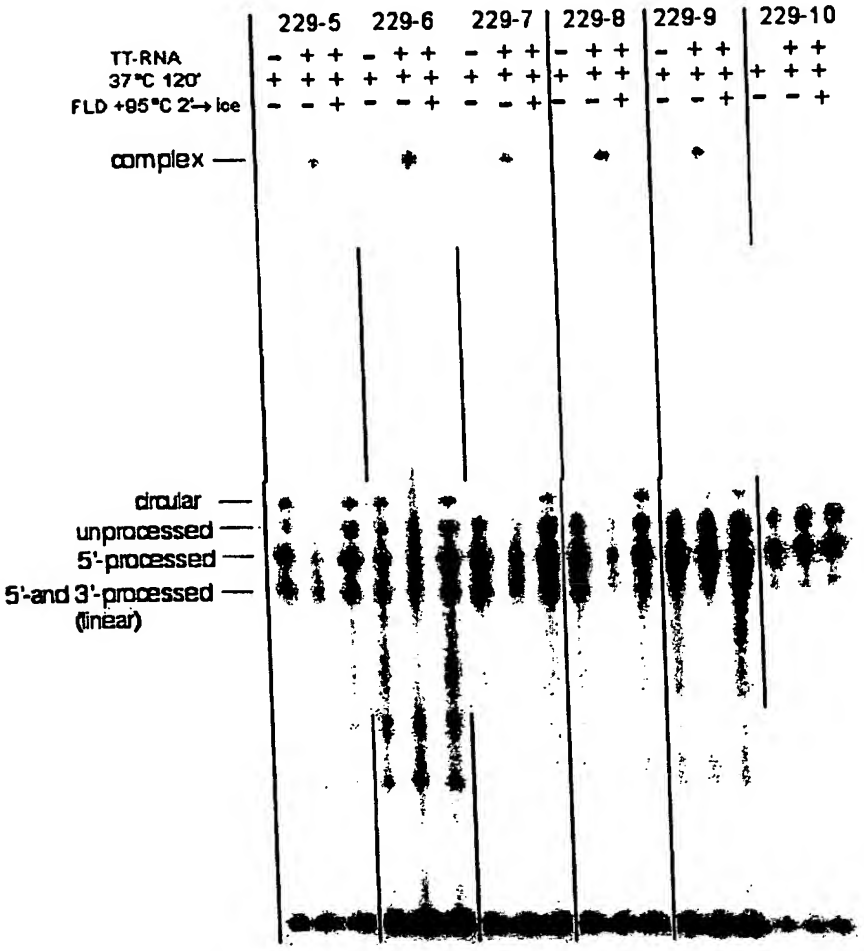

229-5
229-6
229-7
229-8
229-9
229-10
TT-RNA
37°C 120'
FLD +95°C 2'→ ice
complex
circular
unprocessed
5'-processed
5'-and 3'-processed
(linear)

Figure 13

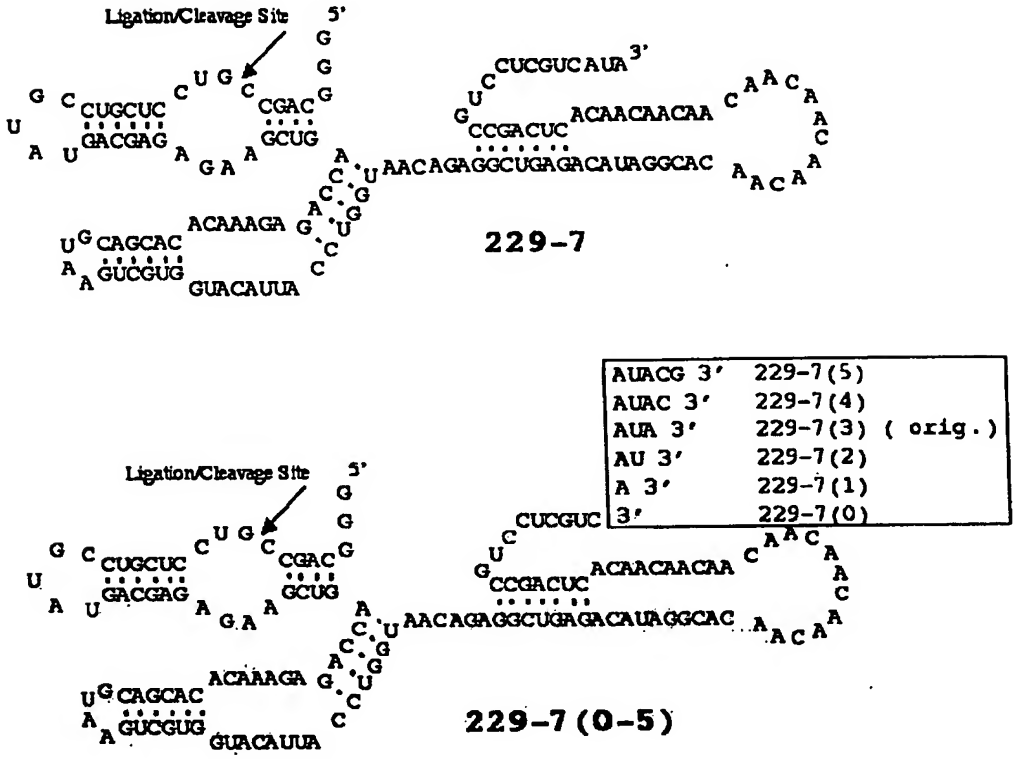

Ligation/Cleavage Site
5'
3'
229-7
AUACG 3' 229-7(5)
AUAC 3' 229-7(4)
AUA 3' 229-7(3) (orig.)
AU 3' 229-7(2)
A 3' 229-7(1)
3' 229-7(0)
229-7 (0-5)

Figure 14

Lasso	229-7(0)			229-7(1)			229-7(2)			229-7(3)			229-7(4)			229-7(5)		
90°C 2'→ice	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
TT-RNA	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+

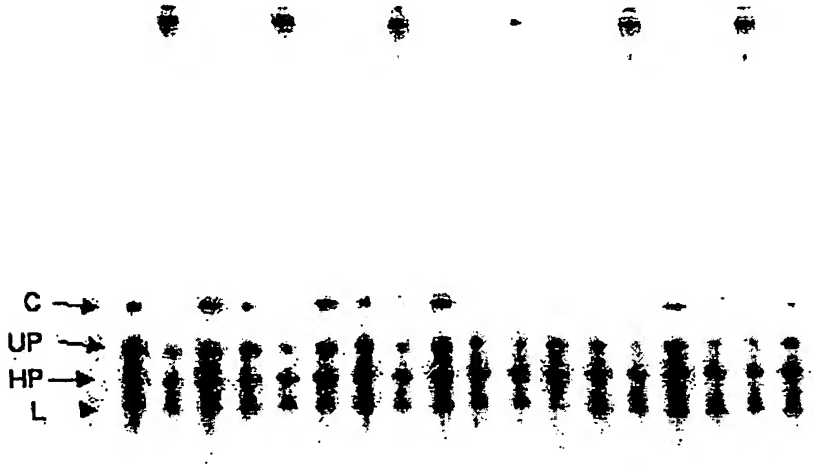


Figure 15

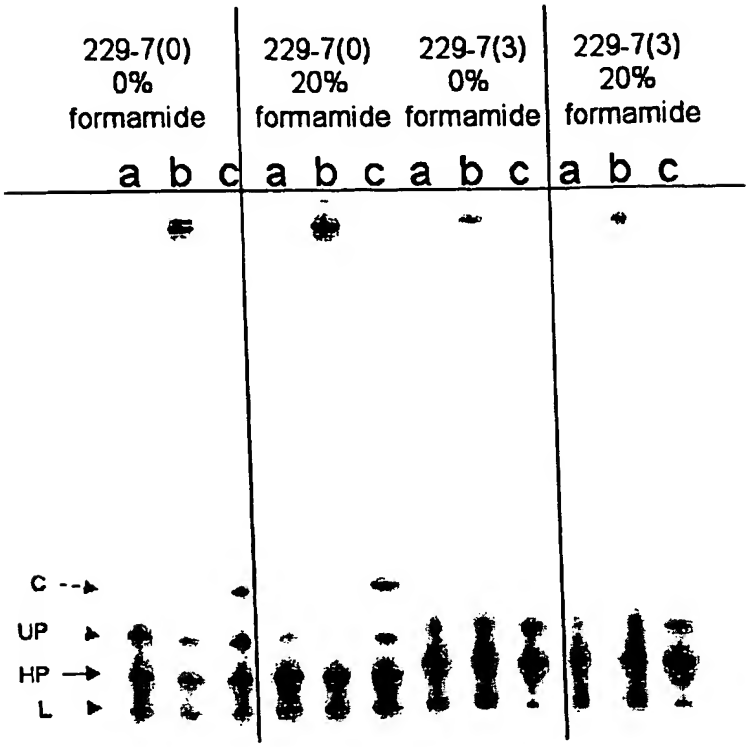
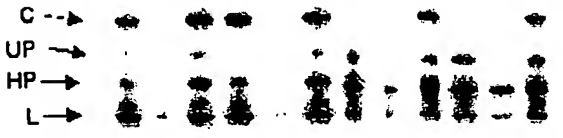

229-7(0)
0%
formamide
229-7(0)
20%
formamide
229-7(3)
0%
formamide
229-7(3)
20%
formamide
a b c a b c a b c a b c
C
UP
HP
L

Figure 16

Lasso	229-5						229-7(0)					
Phys. buffer**	-	-	-	+	+	+	-	-	-	+	+	+
Std. Buffer*	+	+	+	-	-	-	+	+	+	-	-	-
90°C 2' → ice	-	-	+	-	-	+	-	-	+	-	-	+
TT-RNA	-	+	+	-	+	+	-	+	+	-	+	+



*"Standard Buffer":
50 mM Tris-Cl, pH8
10 mM $MgCl_2$
20% formamide

**"Physiological buffer":
20 mM HEPES, pH7.3
140 mM KCl
10 mM NaCl
1 mM $MgCl_2$
1 mM $CaCl_2$

Figure 17

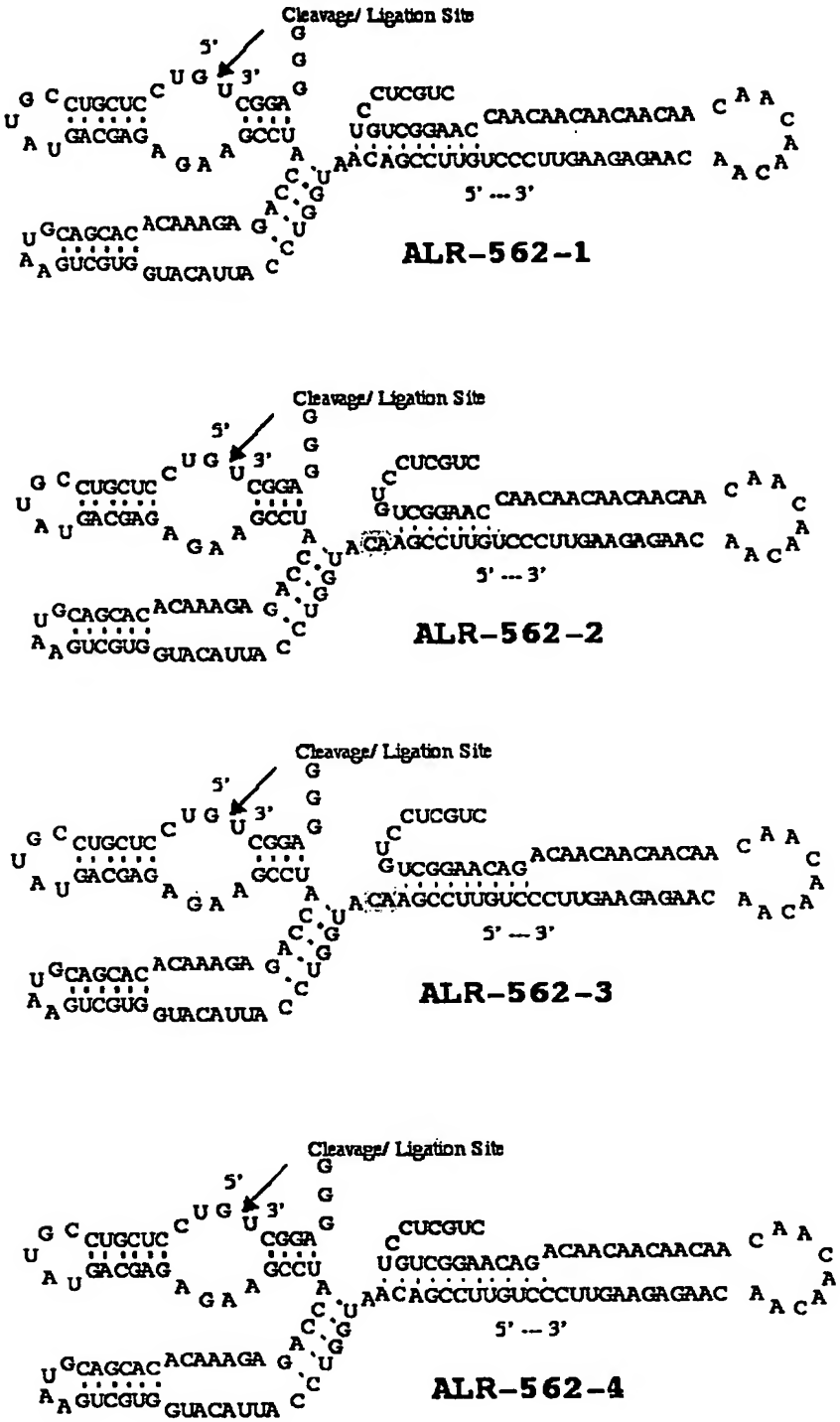

Cleavage/ Ligation Site
5'
3'
5' --- 3'
ALR-562-1
Cleavage/ Ligation Site
5'
3'
5' --- 3'
ALR-562-2
Cleavage/ Ligation Site
5'
3'
5' --- 3'
ALR-562-3
Cleavage/ Ligation Site
5'
3'
5' --- 3'
ALR-562-4

Figure 18

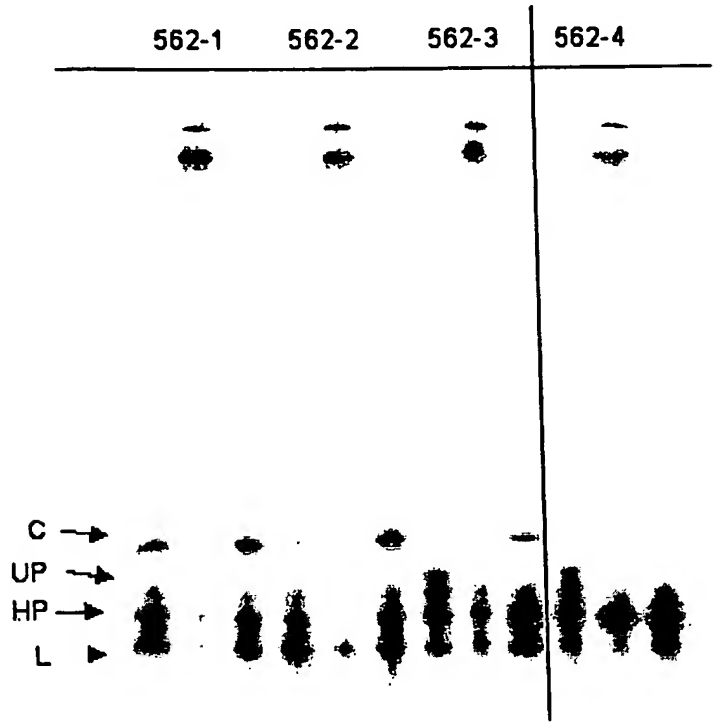


Figure 19

METHOD OF PREPARATION OF RNA CAPABLE OF TARGET-DEPENDENT CIRCULARIZATION AND TOPOLOGICAL LINKAGE

BACKGROUND OF THE INVENTION

[0001] RNA Lassos are catalytic RNA molecules designed to hybridize to target RNAs and ligate their ends together, creating a circle topologically linked (Lasso) around the target RNA or DNA (**Fig. 1**). Topologically linked complexes provide superior stability and specificity over currently available antisense agents and hybridization probes. RNA Lassos can be employed either to inhibit the biological function of target RNA (and DNA) molecules or as probes for detecting the presence of particular sequences associated with a disease. Lasso-target complexes can specifically inhibit gene expression by physically blocking translation of the target RNA, by preventing RNA processing, or by regulating splicing. RNA Lassos can be delivered to cellular targets directly as *in vitro* transcripts; alternatively, they can be expressed *in situ* using plasmid DNA or viral vectors. Where self-ligation of RNA Lassos is made dependent on target-binding, Lassos can also be used for detecting the presence of particular polynucleotide sequences, for example through rolling circle amplification of the circularized Lasso sequence.

[0002] The first generation of RNA Lassos (originally called RNA Padlocks), consisting of self-circularizing hairpin ribozymes and rationally selected antisense sequences, have been successful in inhibiting gene expression in several model systems (Johnston et al., 2000, 2002; Seyhan et al., 2001). Use of all types of antisense agents is complicated by the lack of convenient, reliable methods for selecting effective targeting sequences. Commercialization of antisense-based technologies would be greatly aided by development of efficient, rapid methods for the selection of optimal gene inhibitors from nucleic acid libraries. In the case of Lassos, efficacy would also be aided by a design that maintains Lassos primarily in the linear form until they hybridize to the target mRNA, after which they circularize around the target.

Antisense technology: problems and perspectives.

[0003] Over the past 25 years, antisense oligonucleotides (Lichtenstein & Nellen, 1998; Stein & Krieg, 1998) and nucleic acid ribonucleases (ribozymes and

deoxyribozymes) (Rossi, 1999; Opalinska & Gewirtz, 2002) have been successfully used for artificial suppression and regulation of gene expression through the targeting of messenger or viral RNAs. Quite recently, a new approach using siRNAs (small interfering RNAs) has been used as well (Tuschl, 2002; Zamore, 2002). Thus far, siRNA seems to be the most effective, robust and generally reliable method of translation inhibition, but optimized antisense compounds and nucleic acid ribonucleases can also act as efficiently as siRNA (Braasch & Corey, 2002; Opalinska & Gewirtz, 2002).

[0004] siRNA, antisense agents and ribozymes all can inhibit specific gene expression at the post-transcriptional level involving sequence specific interaction with the target RNA molecules. The RNA interference appears to be more potent and efficient than the other strategies (Jiang & Milner 2002; Aoki et al., 2003). The effectiveness of siRNA is also believed to be less dependent on the accessibility of its target sequence than antisense and ribozyme agents (Paddison & Hannon, 2002). However, there is growing evidence that the siRNA efficacy correlates with the target site accessibility and is strongly dependent on position and sequence of this site (Hohjoh, 2002; Holen et al., 2002; Jiang & Milner 2002; Shi, 2003). Moreover, some RNA targets (including highly-structured RNA, short-living transcripts and viral RNA with high mutation rates) appear to be poor targets for siRNA (Kitabwalla & Ruprecht, 2002; Paddison & Hannon, 2002; Shi, 2003). Also, the optimized antisense agents and antisense RNA can also act as efficiently as siRNA at the same targets albeit not always at the same target sites (Al-Anouti & Ananvoranich, 2002; Braasch & Corey, 2002; Opalinska & Gewirtz, 2002; Vickers et al., 2003). RNA lassos, which can bind to the target stronger than ordinary antisense RNA, can attack less accessible targets and block them through antisense mechanism, and also can potentially induce specific RNA interference by forming short RNA-RNA duplexes similar to siRNA structures. Therefore we are interested in developing library-based strategies to select the optimal RNA inhibitors among RNA lassos and siRNA.

[0005] In contrast to siRNA and nucleic acid ribonucleases that are presumed to inhibit translation through cleavage of their targets, the mechanism of action of antisense agents is not well understood and may actually have little to do with RNase H-assisted cleavage of RNA targets as has long been assumed (Probst & Skutella, 1996; Toulme,

2001; Braasch & Corey, 2002). In fact, many efficient antisense agents function mainly *via* steric blocking of the translation machinery rather than RNase H-mediated cleavage (Probst & Skutella, 1996; Stein, 2000; Toulme, 2001). There is evidence that translation inhibition by antisense agents is not always a result of lowering the levels of target mRNA (Probst & Skutella, 1996). In addition, cleavage of mRNA targets would limit some potential applications of antisense molecules. One such application is the targeting of intron-exon splice junctions so as either to induce a specific alternative splicing event or to prevent the maturation of hnRNA to mRNA (Taylor et al., 1999; Mercatante et al., 2001). Antisense molecules that do not induce cleavage of their targets can also be used as imaging agents for detecting and monitoring gene expression (Pan et al., 1998; Tavitian et al., 1998). An additional benefit of non-cleaving agents is that they can be used for hybridization-based diagnostics, microarrays and affinity capture applications.

[0006] The need for antisense agents that are more potent, selective, robust and reliable is widely recognized, and has led to the development of new agents with higher binding affinity and selectivity as well as schemes for their selection using sequence libraries (Sohail & Southern, 2000). It appears that the affinity of antisense sequences for their target sites (Walton et al., 1999) and the hybridization kinetics of binding (Patzel & Sczakiel, 2000) are the most important factors in the efficacy of antisense agents. Recently, several new nucleic acid derivatives have been developed to generate strong target-binders that do not result in cleavage of the target RNA, including N3'-P5' phosphoramidates, morpholino phosphorodiamidate, 2'-O-methoxyethyl and 2'-fluoro, arabino-nucleic acid (Toulme, 2001; Braasch & Corey, 2002). However, all of these nucleic acid molecules are artificial and, therefore, cannot be expressed by transcription, which is a very good way of providing high intracellular concentration-efficient translation inhibition. DNA and RNA may have less stability *in vivo* than their chemically modified derivatives, but they both can be efficiently and systemically expressed *in situ* from appropriate plasmid DNAs or viral RNA vectors. Moreover, RNA is advantageous over DNA for the following reasons: it can be more efficiently expressed in cells (e.g., using the U6 promoter) than DNA (Noonberg et al., 1994), RNA-RNA complexes are more stable than DNA-RNA (Roberts & Crothers, 1992), and RNA-RNA complexes are not substrates for RNase H-mediated cleavage.

[0007] We have developed a new class of antisense agents using only natural RNA. These agents have the ability to form extraordinarily stable complexes with target RNAs *in vitro* and *in vivo* and eventually could be engineered to be delivered *in vivo* by a viral vector. The concept of using RNA as a therapeutic agent seems unusual (because of "RNA instability" dogma), but it has already been proven to be quite efficient (Sullenger & Gilboa, 2002).

Padlocks, clamps and RNA Lassos.

[0008] RNA Lassos are RNA molecules that can adopt either a linear (open) or a circular (closed) conformation (**Fig. 1**). They contain an antisense sequence embedded in a structure that, upon hybridization to a complementary target mRNA, links the ends of the Lasso. Because of the helical nature of nucleic acid duplexes, a circularized Lasso will be wound around the target strand, topologically connecting two polynucleotides through catenation. This approach is similar to earlier developed DNA clamps (Gryaznov & Lloyd, 1998) and DNA padlocks (Nilsson et al., 1994; Baner et al., 2001) that can form covalent linkages whenever their ends are brought into juxtaposition by hybridization to target polynucleotide. But in contrast to these constructs, the topological linkage can be achieved without either the addition of an exogenous protein ligase as was done for DNA Padlocks or without using terminal, non-nucleotide reactive groups attached to the ends of DNA clamps. Instead, we used a different type of the clamp comprised of natural RNA sequences: a self-ligating hairpin ribozyme (HPR) having distinctive loop-and-stem structural domains (**Fig. 2**). As previously demonstrated, any one of the loops 1-3 can be used for introduction of additional or modified nucleotides without appreciable perturbation of the catalytically-active structure of HPR (Feldstein & Bruening, 1993; Komatsu et al., 1994; Berzal-Herranz & Burke, 1997; Kisich et al., 1999; Fedor, 2000).

[0009] We reasoned that attaching an antisense sequence adjacent to the ribozyme core would allow it to pair with a target mRNA, intertwining the two RNAs. The ends would then refold into the HPR native structure and undergo self-ligation, creating a link between the RNAs that would have the strength of covalent bonding. Thus, we used the ribozyme for a novel purpose--not to cleave the target but to ligate an inhibitor around it.

[0010] To make the ligation target-dependent, we employed allosteric regulation (Porta & Lizardi, 1995; Tyagi et al., 1995; Shih et al., 1996; Asher , 1998; George et al., 1998; Robertson & Ellington, 2000) by introducing a "sensor" inhibitory oligonucleotide sequence partially complementary to either the ribozyme or substrate domain, as well as to the target sequence. The presence of this inhibitory sequence suppresses the catalytic activity of the RNA in the absence of the target. . The sensor sequence is designed so that it hybridizes more strongly to the complementary target sequence than to the enzyme sequence. Upon binding of the ribozyme to the target, the catalytic domain becomes unmasked, and becomes active both as a nuclease and ligase.

RNA Lassos form strong complexes with their targets.

[0011] The previously studied RNA Lasso, ATR1, comprised of a hairpin ribozyme (HPR) core and antisense sequences, was designed to bind to a site in the coding region of mouse tumor necrosis factor alpha (TNF-α) (Johnston et al., 1998).

[0012] Following our standard Lasso preparation protocols, the 133-nt precursor ATR1 (R2) was transcribed from the appropriate PCR-made DNA template. Self-processing of pre-ATR1, which has two internal cleavage sites, by the hairpin ribozyme resulted in semi- (R3a, R3b) and fully processed (R4) RNA species as well as R1, which is the covalently closed, circular form of R4. The lowest electrophoretic mobility of R1 corresponds to a known feature of circular RNA molecules: they migrate in denaturing PAGE more slowly than do their linear forms (Feldstein & Bruening, 1993; Feldstein et al., 1997). The interconverting linear and circular forms of ATR1 were gel-purified and tested with the TNF targets in the gel-shift assays. In control experiments, we used AT RNA, which lacks the ribozyme domain but retains the anti-TNF antisense sequence, and M101 Lasso containing the hairpin ribozyme core but lacking the anti-TNF sequence. For the binding experiments we used two target RNAs, containing either the first 1105 nt (TNF1) or the first 709 nt (TNF2) of the TNF-α mRNA. We demonstrated that ATR1 and TNF1 RNAs could form very strong complexes that were stable enough to be detected by the denaturing PAGE, whereas a complex formed between AT RNA and TNF1 dissociated under the electrophoresis conditions. As expected, no complex

formation was detected between ATR1 and TNF2, which lacks the site complementary to anti-TNF sequence of ATR1, as well as between M101 RNA and both TNF targets.

[0013] We found that unprocessed and semi-processed forms of ATR1 and other first-generation RNA Lassos hybridize with their targets faster than the interconverting linear and circular forms of fully processed Lassos. The circular and linear form of hairpin ribozymes are known to have similar structures, where the RNA ends are held in close proximity to each other by multiple secondary bonds within the catalytic core (Fedor, 2000; Rupert & Ferre-D'Amare, 2001). We suggested that the better hybridization properties of the open Lasso forms might be provided by their looser secondary structures and the presence of free ends that could easily rotate upon binding to the long target. Therefore, we decided to develop second generation Lassos that would be maintained primarily in the linear form until they hybridize to the target, and would then circularize around the target.

[0014] ATR1 complex formation and dissociation was compared in buffers containing and lacking 20% formamide **(Figure 3)**. Comparison of complex formation with ATR1 with and without formamide shows that two discrete gel mobility shifts are observed when there is no formamide in the incubation buffer. When 20% formamide is included in the buffer, only the slower migrating species is observed. In the 0% formamide reaction, the upper band selectively disappears after incubation at 65°C, which coincides with the appearance of the non-circular species of lasso. After incubation at 80°C, the entire lower complex band is dissociated, which coincides with appearance of the circular lasso species. Therefore, the lassos-target complexes have different gel-mobilities depending on whether they are linear or circular, and the complexes with circular lasso are more stable than complexes with the open forms of the lasso.

[0015] One of the second generation Lassos was ALR229 **(Fig. 4)**. The hairpin ribozyme core of ALR229 was modified to match the 6-nt sequence found in the selected target sequence, resulting in internal complementarity between part of the antisense sequence and the ribozyme cleavage/ligation site. This causes misfolding of the ribozyme, inhibiting self-processing and circularization in the absence of target. Upon binding to the target, the inhibitory sequence is displaced and the Lasso can circularize.

Even without optimization of the allosterically regulated elements, ALR229 was shown to be superior over ATR1.

Lasso RNAs can block ribosome scanning and translation more efficiently than antisense RNA.

[0016] After demonstration of enhanced complex stability between the ATR1 Lasso and TNF target, we tested how well this complex was able to block either ribosome scanning of the 5'-UTR (untranslated region) of TNF-α mRNA, or translation elongation in vitro. For this test, we fused the 20-nt TNF target sequence to a luciferase reporter gene and attached a T7 RNA polymerase promoter upstream to create a T7-TNF-Luc cassette. We inserted this cassette into pGL3 Vector (Promega). Run-off transcription of this construct produced a target T7-TNF-Luc RNA that was then pre-hybridized with either ATR1 Lasso or AT antisense molecule, and was finally provided as a template for translation using a rabbit reticulocyte lysate (Promega). Luciferase activity assays revealed that, for an optimal ATR1/target molar ratio of 30:1, ATR1 provided 98% knockdown of the translation, whereas AT was ineffective. Even higher inhibition efficacy (99%) was observed in this translation system for ALR229 Lasso at 20:1 Lasso-/target ratio, even without pre-hybridization with the target (data not shown).

Lasso efficacy in cultured cells.

[0017] To assess Lassos efficacy in cultured cells, we delivered the RNA Lassos, complexed with the cationic lipid LipofectAMINE (Life Technologies/Invitrogen), to a macrophage-like cell line, RAW264.7, testing for their ability to inhibit TNF-α secretion following stimulation of the cells with lipopolysaccharide, LPS (Sigma). Four different Lasso constructs (including ATR1and ALR229) targeted to different sites on the TNF-α mRNA (including both 5'-UTR and coding sequences) were tested. A Lasso construct (M101), lacking any sequence complementary to TNF-α mRNA, was used as a negative control. The TNF accumulation in the media was determined by ELISA assay during the interval 4 to 8 hours after stimulation with LPS at different doses of RNA Lassos (data not shown). Here ALR229 was revealed to be the most effective, inhibiting TNF secretion by 90% at a level (10 μg) that caused no nonspecific toxicity (IC_{50} of 46 nM). NO inhibition of secretion was observed with M101 at similar levels. The inhibitory

activities of the RNA lassos were evident for at least 24 hours after stimulation. In other experiments in which multimers of ALR229 were delivered through a cytoplasmic viral vector based on Semliki Forest Virus (SFV), about 95% inhibition was seen (data not shown).

[0018] Thus, there is evidence that Lassos are more effective than ordinary antisense RNAs both in hybridization and in vitro translation experiments. Also, the Lasso approach permits—for the first time—the successful antisense inhibition of gene expression by antisense sequences targeted to coding regions of mRNAs in the absence of direct target cleavage. The Lassos' efficacy could also be aided by an optimization of allosterically regulated Lassos that provides fast hybridization of the open, linear Lasso to the target followed by the Lasso circularization around the target.

SUMMARY OF THE INVENTION

[0019] We describe novel agents for gene inhibition that have certain advantages over ordinary antisense, ribozyme and RNAi approaches. Allosterically regulated RNA Lassos hybridize to and become topologically linked with complementary RNA targets, creating complexes that effectively block translation. Effective lasso inhibitors can be efficiently derived from specially prepared libraries containing only sequences represented within a defined set of targets.

[0020] Allosterically regulated ribozymes typically contain a "sensor" element that requires specific interaction with an effector molecule to activate (or inactivate) catalytic activity toward a substrate (**Figure 5**). Such ribozymes can be used for detection of a target molecule, where the target plays the role of effector. In one design, an internal base-pairing interaction blocks ribozyme catalysis, and binding of the target competes away this interaction to activate catalysis (**Figure 6**). Such competition may significantly increase the specificity of target recognition. This is supported by recent reports of the high selectivity of oligonucleotide probes containing self-complementary elements to single-nucleotide mismatches or deletions (Li et al., 2002; Bonnet et al., 1999) that suggest that even single-nucleotide mutation (SNP) discrimination is possible.

[0021] The use of a ribozyme that efficiently catalyzes phosphodiester bond formation allows for target-dependent self-circularization. Circularization has several

attractive features: increased resistance of RNA to nucleases, stronger binding around biopolymer targets through topological linkage, and the potential to selectively amplify even trace amounts of circularized species by rolling circle amplification or RT-PCR.

[0022] Here we describe a new class of RNA Lassos that are capable of target-dependent circularization around RNA targets. RNA Lassos contain an antisense sequence attached to a hairpin ribozyme domain that can adopt either a linear or circular conformation. Allosteric regulation was achieved by introducing sensor sequences that bind to a Lasso terminal sequence, preventing self-processing and circularization of the Lasso prior to hybridization with the target RNA. Upon binding to the target RNA, the terminal sequence is released from the sensor sequence, allowing self-processing and ligation of the ends around the target. The resulting topologically linked complexes provide greatly increased binding strength and sequence specificity compared to ordinary antisense RNAs. We have also developed a library-based method to simultaneously optimize both sensor and antisense sequences for efficient binding of different targets.

[0023] Also, a novel approach is described that, starting with libraries of RNA Lassos, provides simultaneous selection of both accessible target sites and optimal design of the Lasso so that circularization is dependent on prior hybridization to the target.

[0024] Individual species of the proposed RNA Lasso libraries differ from one another in two ways. They contain antisense sequences complementary to different segments of the mRNA target, and they also differ in the sequence of the circularizing moieties, which are partially randomized derivatives of the hairpin ribozyme. The antisense sequences constitute a "directed library," which contains all sequences of a given length contained within the targeted gene (or genes), rather than a completely random library **(Figure 7a)**. Directed libraries are of much lower complexity than fully random libraries, allowing coverage of all possible sequences with substantial redundancy of each species. Circularization of Lassos is regulated by introducing inhibitory sequences partially complementary to the hairpin ribozyme core, thus preventing the ribozyme from folding into the self-ligating conformation. The inhibitory sequences are designed so that they hybridize more strongly to the target than to the ribozyme. Thus, binding to the target displaces the inhibitory RNA sequence, and the ribozyme recovers its active conformation.

[0025] These Lasso libraries are exposed to mRNA targets. The resulting strong Lasso-target complexes are isolated, and the circularized Lasso molecules are selectively amplified by RT-PCR using specially designed primers (**Figure 7b**). The resulting PCR products are used as templates for transcription of RNA Lassos for another round of target binding and selection (see **Figure 7c** for schematic). After several rounds, the DNA templates are cloned and sequenced. The selected RNA Lassos sequences are re-synthesized and tested for their ability to tightly and specifically bind the target in vitro, and inhibit translation in both in vitro extracts and in cultured cells. In this way, optimized Lassos are selected in an automatic and straightforward manner and sold as agents for target validation and gene function analysis, antiviral, antibacterial, and gene-therapy drugs.

[0026] Above, the method to select optimal lassos from a fixed hairpin ribozyme domain and semi-randomized antisense sequences and inhibitory sequences has been described. Our selection and amplification scheme (**Figure 7c**) combining rolling-circle amplification with reverse transcription and polymerase chain reaction (RCA-RT-PCR) is universal enough to expand the initial pools of molecules for selection to increase diversity by either partially or fully randomizing any one or more of the three loop regions of the hairpin ribozyme (**Figure 2**). Since any one of the loops 1-3 (**Figure 2**) can be used for introduction of additional or modified nucleotides without appreciable perturbation of the catalytically-active structure of HPR (Feldstein & Bruening, 1993; Komatsu et al., 1994; Berzal-Herranz & Burke, 1997; Kisich et al., 1999; Fedor, 2000), many permutations of Lasso design can be selected using the RCA-RT-PCR scheme. In each case, the Lasso region comprising antisense (or triplex-forming) sequence can be partially randomized (e.g. with a gene-specific directed library) or fully randomized. In addition, the region comprising internal competitive ("inhibitory") sequence that is partially complementary to the antisense (or triplex-forming) sequence can be also fully or partially randomized. Catalytically non-essential residues in the hairpin ribozyme domain can also be partially (semi-random) or fully randomized (random) to increase the initial pool of the Lasso sequence libraries. Below is a partial list of the suggested *combinations* of partial/fully randomized sequences that can be used to generate an initial pool of lassos for selection and amplification by RCA-PCR:

- <defined antisense sequence> with <semi-random internal inhibitory sequence>;
- <defined antisense sequence> with < random internal inhibitory sequence>;
- <semi-random antisense sequence> with <semi-random internal inhibitory sequence>;
- <semi-random antisense sequence> with < random internal inhibitory sequence>;
- < random antisense sequence> with <semi-random internal inhibitory sequence>;
- <random antisense sequence> with < random internal inhibitory sequence>;
- <semi-random HPR sequence> with <semi-random antisense sequence> with <semi- inhibitory sequence>;
- <semi-random HPR sequence> with <semi-random antisense sequence> with <random internal inhibitory sequence>;
- <semi-random HPR sequence> with <random antisense sequence> with <semi-random inhibitory sequence>;
- <semi-random HPR sequence> with <random antisense sequence> with <random inhibitory sequence>.

[0027] Selections with an increased amount of randomized nucleotides in each of the above-mentioned regions can result in an unpredicted, but optimally effective molecular mechanism of allosteric regulation of the Lasso. In each case, the pool of Lassos is incubated with target RNA (or DNA) and Lassos that can circularize in a target-dependent manner are selectively amplified by RCA-RT-PCR as described above. If necessary, fine-tuning of selected sequences can be performed after the selection procedure to further optimize an efficacy of the selected sequences. This may or may not include substitution of individual residues with modified nucleotides if necessary. These modifications include but are not limited by known in art derivatives of nucleobases, sugar residues and internucleotide bonds.

[0028] In addition, sequences in the hairpin ribozyme core domain (i.e. catalytically essential residues) could be altered in a way to improve the efficacy of the cleavage and ligation reactions.

Major RNA Lasso Features:

- Strong complexes of antisense agents with polynucleotide targets can efficiently inhibit gene expression.
- Topological linkage enhances stability of polynucleotide complexes.
- RNA Lasso sequence encodes antisense-ribozyme that does not cleave target but can: cut Lasso out of longer transcript; convert open linear Lasso to closed circular form.
- Lasso circularization around target creates topological linkage.
- Target-dependent circularization of Lassos can optimize formation of the topological linkage.
- Design of allosterically regulated ribozyme domain should allow the Lasso circularization only after, and as result of, Lasso-target hybridization.
- Use of partially randomized Lasso sequences and SELEX schemes based on the Lasso circularization allows simultaneous selection of optimal target sites and Lasso sequence variants.
- Use of gene-specific directed libraries incorporated into Lasso structure makes the selection experiments more cost- and time-effective than use of completely randomized sequences.

DESCRIPTION OF THE DRAWINGS

[0029] **Figure 1.** Schematic presentation of RNA Lassos binding to RNA target resulting in formation of a topologically linked complex.

[0030] **Figure 2.** Schematic representation of consensus structures of the hairpin ribozyme (HPR). HPR is present in the sequence of the minus strand of Tobacco ringspot virus satellite RNA (sTRSV). The site-specific RNA cleavage induced by the ribozyme generates fragments having 2',3'-cyclic phosphate and 5'-OH termini. HPR can efficiently ligate those ends and can exist as linear and circular forms that interconvert. The internal equilibrium between circular and linear forms depends on the relative stability of the cleaved and ligated forms. Dots represent any nucleotide (A, U, G or C), dashes represent required pairings, V is 'not U' (A, C, or G), Y is a pyrimidine (U or C),

R is a purine (A or G), B is 'not A' (U, C or G), H is 'not G' (A, C or U) (Berzal-Herranz & Burke, 1997).

[0031] **Figure 3.** Circularized lassos form stronger complexes with target RNA than linear Lassos. Complexes were formed at 37°C for 2 hrs. Buffer: 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8) with or without formamide as indicated. Displacement Procedure: Complexes with one volume of loading buffer added have been incubated 2 min at temperature indicated followed by ice-cooling before loading in the gel (PAGE: 6% PAGE/8M UREA)

[0032] **Figure 4.** Putative structure of fully-processed ALR229 shown in complex with TNF-α mRNA target.

[0033] **Figure 5.** Allosteric regulation of a lasso hairpin ribozyme can be achieved by introducing inhibitory internal base pairing sequences that bind to a Lasso terminal sequence, preventing self-processing and circularization of the Lasso prior to hybridization with the target RNA.

[0034] **Figure 6.** Schematic of processing scheme of Lasso hairpin ribozyme

[0035] **Figure 7.** Putative structure of ARDL-10N Lasso library containing partially randomized ribozyme and inhibitory sequences, and a directed antisense library. a) Unprocessed Lasso. (b) Fully processed Lasso with PCR primers aligned to amplify the covalently closed circular form exclusively. N represents any nucleotide (A, G, C and U). Defined GC sequences in the inhibitory and antisense sequences were preserved to decrease the complexity of the library. The GC dinucleotide sequence is present in the majority of the potential target sites, and it can be located at any place within these sites. (c) Detection and amplification of circularized Lassos by Rolling Circle Amplification and PCR.

[0036] **Figure 8. Putative structure of unprocessed ALR229-5N** and its derivatives, ALR229-5, 229-6, 229-7, 229-8, 229-9 and 229-10 which differ in the length of the inhibitory element, respectively having 5, 6, 7, 8, 9 and 10 nucleotides complementary to the Lassos antisense domain. The inhibitory sequence includes 5 nt immediately adjacent to the ribozyme cleavage/ligation site. ALR229-5N is a library of Lassos having all four nucleotides (A, G, C and U) at each of the N positions.

[0037] **Figure 9. Putative scheme of ALR229-10 Lasso self-processing and binding to the TNF target.** The unprocessed pre-Lasso undergoes a self-cleavage at the 5'-end. The self-cleavage of its 3'-end is inhibited by an intramolecular base-pairing of the 10-nt long inhibitory element with the Lasso's antisense domain. The inhibitory sequence includes 5 nt immediately adjacent to the ribozyme cleavage/ligation site. Upon binding to the target, this 5-nt sequence will be released that allows the Lasso's 3'-end cleavage. The fully processed Lasso, bound to the target, then can undergo circularization.

[0038] **Figure 10. Binding of ALR229-5-6-7-8-9-10 Lassos with TNF2 (709 nt) and TNF-20 (20-nt) target RNAs reveal the allosteric regulation of the Lassos self-processing and very strong complex formation with the long target.** The trace amounts of the internally ^{32}P-labeled Lassos were incubated in 10 mM $MgCl_2$ / 50 mM Tris-Cl (pH 8) for a total of 120 minutes at 37°C, either alone (lanes 1) or with non-radioactive 0.4 μM TNF-20 (lanes 2), or with 0.4 μM TNF2 (lanes 3-5). Lanes 4 are the same as lanes 3 but chased with a 14-fold excess of 20-nt competitor antisense RNA, anti-TNF-20 over TNF2. Lane 5 is the same as lane 3 but chased with 7-fold excess of competitor sense TNF-20 (20-nt) over TNF2. Samples were analyzed by 6% denaturing PAGE. Anti-TNF-20 is identical to the antisense sequence incorporated into the Lassos. TNF-20 corresponds to the sequence of TNF-α mRNA targeted by these Lassos. Abbreviations: S is PAGE start; LLT are Lasso complexes with the long target (TNF2); LST are Lasso complexes with the short target (TNF-20); CL are the circular forms of fully-processed Lassos; UPL are unprocessed pre-Lasso transcripts; 5PL are 5'-end semi-processed pre-Lassos; L are fully-processed, linear Lasso.

[0039] **Figure 11. Self-processing of ^{32}P-internally-labeled allosterically-regulated lassos in buffer containing or lacking 20% formamide.** Each lasso was incubated in either 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$ (- lanes) or 50 mM Tris-HCl, 10 mM $MgCl_2$, 20% formamide (vol/vol) (+ lanes) for 120 minutes at 37°C. One volume of Loading Buffer containing 90 % formamide, 10 mM EDTA was added to each sample. The samples were then loaded onto a 6% PAGE gel containing 8M urea and 0.5X TBE. C, circular lasso, UP, unprocessed lasso, HP, Half processed lasso, L, 5' and 3' processed linear lasso.

[0040] **Figure 12. Binding of ^{32}P-internally-labeled allosteric lassos to targetRNA in buffer containing 50 mM Tris-Cl, pH8, 10 mM $MgCl_2$, 20% formamide.** Lassos were incubated in the buffer indicated for 120 minutes at 37°C either alone (-) or with 1.4 mM target RNA in excess over lassos (+). One volume of Loading Buffer containing 90 % formamide, 10 mM EDTA was added to each sample. The samples were then loaded onto a 6% PAGE gel containing 8M urea and 0.5X TBE. C, circular lasso, UP, unprocessed lasso, HP, Half processed lasso, L, 5' and 3' processed linear lasso.

[0041] **Figure 13. Analysis of target-dependent circularization of lassos 229-5, -6, -7, -8, -9, and –10.** ^{32}P-internally-labeled allosteric lassos to targetRNA were incubated in buffer containing 50 mM Tris-Cl, pH8, 10 mM $MgCl_2$, 20% formamide for 120 minutes at 37°C, either alone or with 1.4 mM target RNA in excess over lassos as indicated. Following incubation, one volume of Loading Buffer containing 90 % formamide, 10 mM EDTA was added to each sample. Half of the lasso-target complex sample was heated at 90°C for two minutes followed by immediate transfer to ice. The samples were then loaded onto a 6% PAGE gel containing 8M urea and 0.5X TBE. C, circular lasso, UP, unprocessed lasso, HP, Half-processed lasso, L, 5' and 3' processed linear lasso.

[0042] **Figure 14. Sequences and secondary structures of allosterically regulated lasso 229-7 and its derivatives.**

[0043] **Figure 15. Analysis of target-dependent circularization of 229-7 series of allosterically regulated lassos.** ^{32}P-internally-labeled allosteric lassos to targetRNA were incubated in buffer containing 50 mM Tris-Cl, pH8, 10 mM $MgCl_2$, 20% formamide for 120 minutes at 37°C, either alone or with 1.4 mM target RNA in excess over lassos as indicated. Following incubation, one volume of Loading Buffer containing 90 % formamide, 10 mM EDTA was added to each sample. Half of the lasso-target complex sample was heated at 90°C for two minutes followed by immediate transfer to ice. The samples were then loaded onto a 6% PAGE gel containing 8M urea and 0.5X TBE. C, circular lasso, UP, unprocessed lasso, HP, Half-processed lasso, L, 5' and 3' processed linear lasso.

[0044] **Figure 16. All samples were incubate in buffer containing 50 mM Tris-HCl, pH8, 10 mM MgCl2, with or without formamide as indicated.**

[0045] **a** Lasso only (no target). **b** Lasso + target (LT-complex) incubated 37°C 120'. **c** LT-complex incubated 90°C 2', immediately to ice before loading. The samples were then loaded onto a 6% PAGE gel containing 8M urea and 0.5X TBE. C, circular lasso, UP, unprocessed lasso, HP, Half processed lasso, L, 5' and 3' processed linear lasso.

[0046] **Figure 17 Lasso-target complex formation and target-dependent circularization comparison between standard buffer and physiological buffer.** ^{32}P-internally-labeled allosteric lassos were incubated in the buffer indicated for 120 minutes at 37°C either alone (-) or with 1.4 uM cold target RNA in excess over lassos (+). One volume of Loading Buffer containing 90 % formamide, 10 mM EDTA was added to each sample. Half of the lasso-target complex sample was heated at 90°C for two minutes followed by immediate transfer to ice. The samples were then loaded onto a 6% PAGE gel containing 8M urea and 0.5X TBE. C, circular lasso, UP, unprocessed lasso, HP, Half processed lasso, L, 5' and 3' processed linear lasso.

[0047] **Figure 18. Designed allosterically regulated lassos with antisense target sequence to nucleotides 562-583 of murine TNF□.**

[0048] **Figure 19. Target binding and target-dependent circularization for ALR-562 series lassos.** For each lasso in series, Lane 1 is lasso only incubated at 37°C for 120 min., Lane 2 is lasso + TT-280 RNA incubated at 37°C for 120 min., and lane 3 is same as lane 2, but incubated for an additional 2 min. at 95°C and place immediately on ice prior to loading. C, circular lasso, UP, unprocessed lasso, HP, Half processed lasso, L, 5' and 3' processed linear lasso.

DESCRIPTION OF THE EXPERIMENTAL DESIGN, METHODS OF PREPARATION AND USE THEREAFTER OF RNA LASSOS

[0049] To improve efficacy of lasso interaction with target RNA and circularization thereby creating a topological link between lasso and target, we have developed a method to make the lasso circularize only after it binds to target RNA using a unique design of allosteric regulation. It was found that for previously characterized RNA Lassos (formerly known as RNA padlocks), the unprocessed and semi-processed

forms hybridized to target RNA faster than the inter-converting linear and circular forms of the fully processed Lasso. The circular and linear forms of hairpin ribozymes are known to have similar structures, such that the 5'- and 3' ends of the RNA are actually in close spatial proximity and are stabilized by multiple hydrogen bonds within the catalytic core. Therefore, we decided to develop improved Lassos that would be maintained primarily in the linear form until they hybridize to the target, and would then circularize around the target.

[0050] To make the Lasso processing and ligation target-dependent, we employed allosteric regulation by introducing an inhibitory oligonucleotide sequence complementary (typically, 5 to 10 nucleotides in length) to the hairpin ribozyme domain and to the target sequence. A series of allosterically regulated lassos was designed and synthesized to target the 229-249 region of murine TNFα (**Fig. 7**). The presence of this inhibitory sequence suppresses the catalytic activity of the RNA in the absence of the target. Upon binding of the Lasso to the target, the catalytic domain is unmasked and becomes active both as a nuclease and ligase.

[0051] For further improvement of Lasso efficacy, we designed and optimized an allosterically regulated lasso based on ALR229 that would circularize in a target dependent manner. First, we modified the structure of hairpin ribozyme core (see **Figs. 4** and **8**) according to Esteban et al. (1997) to make it more efficient in self-processing. Second, we prepared six Lasso derivatives (ALR229-5, 229-6, 229-7, 229-8, 229-9 and 229-10) that were different in the length of the inhibitory elements (i.e. having 5, 6, 7. 8, 9 and 10 nucleotides complementary to the Lasso antisense domain) (**Fig. 8**). All of these inhibitory sequences include 5 nucleotides immediately adjacent to the ribozyme cleavage/ligation site. The longer the complementarity between the inhibitory element and the Lasso's antisense domain, the stronger the internal inhibition of the circularization prior the target binding. Upon binding to the TNF target, the substrate sequence could be released and Lasso circularized as schematically shown for ALR229-10 in **Fig. 9**.

[0052] The Lassos were constructed using 4 overlapping DNA oligonucleotides. Briefly, 2 overlapping, internal oligos are annealed and overhangs are filled in by Klenow extension. The other 2 primers are used to amplify the rest of the sequence by PCR.

The Lassos are then *in vitro* transcribed using T7 RNA polymerase and desalted over a G50 micro-spin column.

[0053] Detailed example of Lasso 229-7 synthesis is described below. Two partially complementary overlapping oligonucleotides (all oligonucleotide sequences are shown in 5'-3' direction):

CGTCCGTATGACGAGAGAAGCTGACCAGAGAAACACACGACGTAAGTCGTG GTACATTACCTGGTAACAGAGGC (74 nt)

TGTTGTTGTTGTTGTTGTTGTGCCTATGTCTCAGCCTCTGTTACCAGGTAATGT ACCACGACTTACGTC (69 nt)

are annealed at 80°C for 5 minutes and allowing to slowly cool to room temperature over the course of an hour. The oligos are then filled in by Klenow extension to create a completely double stranded template. Primers to amplify this sequence using PCR and add on a T7 promoter sequence are as follows:

TAATACGACTCACTATAGGGCAGCCGTCCTCGTCCGTATGACGAGAGAAGC (51 nt) and TATGACGAGGACGGCTGAGTGTTGTTGTTGTTGTTGTTGTGC (45 nt). The PCR product is gel purified on an agarose gel. This gel-purified fragment is used as a template for in vitro run off transcription by standard methods. ^{32}P is internally incorporated in the transcribed lassos using [^{32}P-α]CTP in the transcription mixture. The transcription reaction is desalted on a Sephadex G-50 spin column and stored at –20°C until further use.

[0054] Lassos ALR229-5-6-7-8-9-10 were transcribed from DNA templates and tested in binding experiments with the long TT-RNA target (671 nt) as well as with the short TNF-20 RNA comprising just 20-nt of target TNF sequence (**Fig. 10**). As expected the Lasso transcripts (pre-Lassos) underwent self-cleavage at their 5'-ends during transcription, whereas the cleavage of their 3'-ends was inhibited (see **Fig. 9**). Interestingly, ALR229-9 and ALR229-10 self-cleave their 5'-ends less efficiently (during both transcription and incubation in the presence of TNF2, but not if incubated alone or in the presence of TNF-20) than the other Lassos. We found that inhibition increased with increasing length of the inhibitory elements (**Fig. 10**, lanes 1). We demonstrated that Lassos ALR229-6-7-8-9-10 indeed underwent allosteric regulation upon binding to

the target sequence. The target binding allowed the ribozyme to complete self-processing, yielding fully processed linear Lassos (**Fig. 10**, lanes 2).

[0055] Typical binding assays were performed using internally radiolabeled lassos are incubate with excess of target RNA at 37°C, 120 minutes in one of three buffers: (i) 50 mM Tris-HCl, pH8, 10 mM $MgCl_2$; (ii) 50 mM Tris-HCl, pH8, 10 mM $MgCl_2$,, 20% formamide volume/volume; (iii) 20 mM HEPES, pH 7.3, 140 mM KCl, 10 mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$. One volume of loading buffer is added containing 90 % formamide, 10 mM EDTA, 0.01% bromophenol blue, 0.01% xylene cyanol. Samples are loaded on 6% PAGE/8M urea/0.5x TBE gels and are electrophoresed at 11 Watts for approximately two hours. The gel is dried and either directly scanned by phosphorimager or exposed to X-ray film.

Monitoring target-dependent circularization:

[0056] An aliquot of the lasso-target complex is heated in loading buffer for 2 minutes at 90°C and then place immediately on ice to prevent complex re-hybridization prior to loading on a denaturing gel.

[0057] We showed that the ALR229-5-6-7-8 Lassos bound the long target stronger and more efficiently than the short one, and also that the Lasso-TNF2 complexes were more stable than Lasso-TNF-20 under the conditions of denaturing PAGE (**Fig. 10**, lanes 2-3). Overall, ALR229-6-7-8 Lassos were the most effective at target binding. The superior stabilities of the Lasso-TT-RNA complexes were also confirmed by chase experiments. We found that short sense or antisense RNAs, (identical or complementary to the TNF-α target site) could not displace the long target from the Lasso-TNF2 complexes (**Fig. 10**, lanes 4-5). We think that the increased stability of Lasso-TT-RNA complexes is a result of an interlocking between the two RNA secondary structures still present in TT-RNA (but not in TNF-20) even in the denaturing gel conditions.

Effect of 20% formamide in lasso self-processing and target binding ability.

[0058] The effect of 20% formamide on the efficiency of lasso processing and target-binding reaction was tested (**Figs. 11 and 12**). Although there was a dramatic effect on the self-processing of the lasso when lasso was incubated in the binding buffer

(**Figure 11**), inclusion of formamide in the binding reaction did not improve the ability of the lasso to gel-shift TT-RNA (**Figure 12**). Inclusion of formamide in the incubation buffer, resulted in the conversion of unprocessed lasso to either half-processed or fully processed forms (**Figure 11**). In general, for the lassos with shorter regulatory elements such as 229-5, the conversion to linear form occurs even when target is not present. However, lassos 229-9 and 229-10 still are unable to be gel-shifted by TT-RNA, consistent with previous results where formamide was not included in the incubation buffer.

Assay for target dependent circularization

[0059] By displacing Lassos bound to TT-RNA using highly denaturing conditions (60% formamide/10 mM EDTA, 95°C), we detected the circularization of ALR229-7-8-9 Lassos induced by target binding.

[0060] Lasso-target complexes are dissociated if exposed to 95°C for 2 minutes after addition of 1 volume of formamide loading dye, containing 90% formamide, 10 mM EDTA, followed by transfer to ice. Once dissociated the lasso species present before and after incubation with target RNA can be analyzed by denaturing PAGE. This was done for Lassos 229-5 through 229-10. Lassos 229-7, 229-8, and 229-9 show an accumulation of *circular* Lasso species that was not present when the Lasso was incubated without target. 229-5 and 229-6 show some circular species before incubation with target and do not show allosteric regulation. After heat treatment, circular form remains, but not more than was originally present. As mentioned previously, 229-10 does not form complex with target RNA. **(Figure 13)**

Optimization of the 3'-end of Lassos.

[0061] To test the effect of extended complementarity of the 3'-end to helix 1 of the HPR domain of the allosterically regulated lassos, a series of lassos were designed with altered 3'-ends (229-7(0-5)) **(Figure 14)**. I have assayed 229-7(0-5) lassos for ability to bind to target RNA and for circularization upon complex formation **(Figure 15)**. All of the lassos were able to bind to target TT-RNA efficiently, but the amount of circularization upon target binding decreased as the length of the complementarity of the

3'-end of the lasso increased. Lassos 229-7(0,1, and 2) show an improvement in the amount of lasso that has circularized after being incubated with TT-RNA than the original 229-7(3). **Figure 16** shows a comparison between lassos 229-7(0) and 229-7(3) in buffer containing or lacking 20% formamide. Circular RNA is produced even under the more denaturing conditions. Decreasing the length of the complementarity of the 3'end of the lasso does promote a higher level of circularization.

"Physiological" buffer studies

[0062] I have tested the interaction between lasso and target under buffer conditions that are considered to be more physiologically relevant than our standard buffer conditions (50 mM Tris-Cl pH8, 10 mM $MgCl_2$, 20% formamide). The more physiological buffer tested is a buffer that contains 20 mM HEPES, pH 7.3, 140 mM KCl, 10 mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$. Under these conditions, lassos 229-5b and 229-7(0) bind efficiently to target TT-RNA and show similar amounts of circularization after incubation at 37°C for 120 minutes and subsequent complex displacement by 95°C treatment as is observed for the standard buffer (**Figure 17**). Circularization of 229-7(0) after incubation with the target RNA demonstrates that the lasso is able to ligate in conditions with low divalent cation concentration. It should be noted that for the allosterically regulated lasso 229-7(0), circularization is completely dependent on the presence of target whereas for the non-allosterically regulated lasso 229-5b, circularization occurs in the absence of target RNA.

Lassos directed at alternate target sites

[0063] A series of lassos that are allosterically regulated was designed to target the TNF 562-583 sequence, the same target sequence used in the original ATR1 lasso. The inhibitory sequences in these lassos ranges between 7 and 10 base pairs (**Figure 18**). The lassos were assayed for target binding and target dependent circularization (**Figure 19**). All of these new lassos circularize when incubated with target RNA, and all bind to target RNA strongly. Curiously, 562-2 (7 basepair) is more allosterically regulated than ALR-562-1 lasso even though it has two fewer potential base pairs.

[0064] 562-2, which has a 7 basepair masking sequence, is interesting because it circularizes very efficiently only when incubated with target RNA. However, the mechanism of allosteric regulation is different than other allosterically regulated lassos because it almost fully processes to linear (5' *and* 3' processed) upon incubation in standard buffer without target. The equilibrium of the lasso processing is such that the 3'-end is able to process efficiently but ligation does not occur until the lasso is incubated with the target RNA. Therefore, different allosteric regulation mechanisms are possible although the design of the inhibitory element is the same.

Selection of the optimized Lasso from the partially randomized libraries.

[0065] We developed a method for detecting and amplifying only Lassos in their covalently ligated circular form. To detect the circularization of Lasso RNAs, we design and order primers for RT-PCR (similar to shown in **Fig. 7b**) that will amplify only the circular Lassos.

[0066] Primer 1 is designed to be complementary to the 5'-end of the 5'-processed Lasso. In the reverse transcription (RT) reaction, Primer 1 selectively extends only circular Lassos, yielding single-stranded DNA multimers of the Lasso sequence (rolling circle amplification, RCA) under the conditions described by Seyhan et al. (2002). One or two additional primers (Primer 2 and Primer 3) are used to amplify the RCA product by PCR and to restore the T7 promoter sequence at the Lassos 3'-end so that the products may be transcribed in vitro. Since this PCR reaction sometimes yields multiple products, the DNA fragment corresponding to the monomer Lasso sequence is gel-purified, and then re-amplified by PCR. The resulting DNA template is used for transcription of selected Lasso RNAs.

[0067] The internally ^{32}P-labeled Lasso RNAs are incubated at 37°C with TNF2 target (709-nt fragment of TNF mRNA) in buffer containing 50 mM Tris-Cl (pH 7.5) / 10 mM $MgCl_2$/20% formamide (standard binding conditions). The resulting complex is isolated by denaturing PAGE. The band corresponding to the Lasso-target complex is localized on the gel by autoradiography, then excised and eluted. The eluted Lasso caught on the target is then amplified by RT-PCR exactly as described above.

Synthesis of a small Lasso library comprising a rationally designed hairpin ribozyme domain, a randomized inhibitory element and a defined antisense sequence.

[0068] A mini-library is synthesized of ALR229-5N Lassos (**Fig. 8**), which contain the rationally designed hairpin ribozyme domain, a defined antisense sequence and a hemi-random inhibitory element. DNA templates for the mini-library transcription are prepared using four DNA primers. First, two overhanging primers that code for the internal Lasso region are annealed and extended by DNA Polymerase I (Klenow fragment). Two additional primers that include the flanking Lasso sequences are used to extend and amplify the resulting DNA template by PCR. The prepared Lasso DNA library, containing a 5 bp randomized region, is then transcribed into the Lasso RNA library. Lasso RNAs are desalted by gel-filtration (on a G-50 micro-spin column) and incubated with TNF2. The Lasso-target complexes are isolated, and the circularized Lasso RNAs are passed through several rounds of selection as described above. After the last round of selection, 20 resulting DNA fragments are cloned and sequenced. The obtained sequences are compared to the related sequences of ALR229-5-6-7-8-9-10 and statistically analyzed.

Design and preparation of a Lasso DNA cassette to encode a Lasso RNA library. Design the sequence of the RNA Lasso scaffold to contain a partially randomized hairpin ribozyme domain, a randomized inhibitory element to select for target-dependent circularization, and directed antisense sequences.

[0069] Using the 229-5N Lasso mini- library as a scaffold, we design an RNA Lasso Library comprising partially randomized ribozyme and inhibitory sequences, and a directed antisense library (**Fig. 7a**). Restriction sites (*XhoI* and *BamHI*) flank either side of a 20-nucleotide antisense cassette sequence, which is supplied by the directed library. The Lasso contains a 10 nucleotide randomized region downstream of the BamHI site and loop, which constitutes the variable allosteric regulatory element that is optimized through iterative rounds of selection and amplification. The 5'-end of the ribozyme core is also partially randomized to allow for proper processing of the 3'-end of the Lasso molecules induced by the binding to the target. Throughout the Lasso, nucleotides essential to hairpin ribozyme activity (see **Fig. 2**) have been preserved.

[0070] Based on the structure of the RNA Lasso library, we design and synthesize the DNA library cassette encoding the ribozyme, inhibitory sequences, and the restriction sites allowing the insertion of the directed antisense libraries in desirable orientation. This DNA library cassette is prepared in two halves to prevent PCR amplification of cassettes without the directed library insert. The first half contains the T7 promoter sequence, the hairpin ribozyme domain, and the XhoI restriction site. The second half includes the BamHI site, the inhibitory element and the 3'-end of the lasso. Each template segment contains an arbitrary sequence adjacent to the restriction sites to enable efficient digestion.

[0071] Preparation of directed DNA libraries encoding up to 20-nt-long sequences complementary to TNF-α, flanked by restriction sites to allow insertion into the DNA library cassette, using Somagenics' proprietary methods for preparing directed antisense libraries as follows. Design and prepare two hemi-random probes consisting of a defined sequence (PCR primer and restriction site sequences) and 10-nt randomized regions. Hybridize the hemi-random probes to TNF-α cDNA and ligate the adjacent probes with DNA ligase. Amplify the ligation products by PCR, followed by digestion them with the appropriate restriction enzymes.

[0072] The target cDNA, comprising the sequence of target mRNA, is prepared using plasmid PGEM-4/TNF, encoding MuTNF-α, by asymmetric PCR with an unmodified primer. Alternatively, regular exponential PCR with biotinylated primer can be used with subsequent ccDNA strands separation on streptavidin magnetic beads Dynabeads M-280 Streptavidin (Dynal; 2000l). On the first step the dsDNA will be immobilized on the beads due to biotin-streptavidin binding. The mixture is then be heated to separate the DNA chains: non-biotinylated one appear in the flow-through, while the biotinylated remain attached to the beads.

[0073] Two hemi-random DNA probes are designed and synthesized, comprising sequences of the defined PCR primer (20 nt) and restriction sites, Xho I & Bam HI (6 nt), with a randomized region (10 nt). Also, masking oligonucleotides that are complementary to the constant regions of the probes are prepared. The hemi-random probes (with constant regions protected with masking oligos) are hybridized to TNF-α

cDNA and the adjacent probes are ligated by T4 DNA ligase at 25-40°C as described by Kazakov et al (2002). The ligated probes are amplified by PCR using specific primers.

[0074] The Lasso DNA library cassettes are digested with appropriate restriction enzymes, and ligated with the digested directed antisense library. Amplify the ligated product by PCR and transcribe the RNA Lasso library.

[0075] To combine the directed library with the DLC halves, two halves of the DNA library cassette and the directed library species are digested with the appropriate restriction enzymes to generate cohesive ends. The digested products are gel-purified and ligated by DNA Ligase. The ligated product is PCR-amplified using primers that are specific to the full-length ligated product. The amplified DNA molecules are gel-purified and used as templates for transcription of the Lasso RNA Library schematically presented in **Fig. 7b**.

Selection of the RNA Lassos best able to quickly bind and form topological links with specific targets.

[0076] Incubate Lasso RNA libraries with target, isolate complexes on affinity columns, selectively-amplify circularized Lassos within complexes, and transcribe RNA from the PCR products. After several additional rounds of selection, surviving members of the library are cloned, sequenced, re-synthesized and tested in the binding assays

[0077] The RNA Lasso libraries are incubated with the target TNF-α mRNA. The resulting complex is isolated using biotinylated ccDNA complementary to TNF-α mRNA immobilized on streptavidin-coated magnetic beads as described previously (Deyev et al., 1984; Stiege et al., 1988; Dynal, 2000). To prevent enrichment of unrelated RNAs (false-positive) that can non-specifically bind beads and undergo self-circularization in a target-independent manner, we perform a counter-selection with the blank magnetic beads or non-specific RNA target (e.g., biotinylated IL-1 ccDNA). After a brief incubation, the beads are washed intensively to remove non-bound and non-specifically bound molecules. Then Lassos specifically bound to the target RNA are eluted (**see Figure 20**).

[0078] The eluted Lassos caught on the TNF target are amplified by RT-PCR as described above. If amplification proves difficult on the Lasso-target complex, then the Lasso-target complex is dissociated prior to primer extension under highly denaturing conditions that retain the integrity of circularized Lasso. After the first round of

selection, the Lasso DNA library is transcribed into the Lasso RNA library. Lasso RNAs are desalted by gel-filtration (on a G-50 micro-spin column) and incubated again with the target TNF-α mRNA. The Lasso-target complexes are isolated as described above, and the circularized Lasso RNAs are passed through several additional rounds of selection.

[0079] After the last round of selection, around 50-100 resulting DNA fragments are cloned and sequenced. The obtained sequences are assigned to the TNF target sequences and statistically analyzed.

[0080] These selected, individual RNA Lassos are transcribed and tested in binding (kinetics, affinity) experiments. Each selected Lasso is incubated with TNF target under standard binding conditions. The strong complexes are identified by analysis on denaturing PAGE.

BIBLIOGRAPHY

Al-Anouti, F., Ananvoranich, S. (2002) Comparative analysis of antisense RNA, double stranded RNA, and delta ribozyme-mediated gene regulation in Toxoplasma gondii. *Antisense Nucleic Acid Drug Dev.* **12**, 275-281.

Aoki, Y., Cioca, D.P., Oidaira, H., Kamiya, J., Kiyosawa, K.. (2003) RNA interference may *be more potent than antisense RNA in human cancer cell lines. Clin. Exp. Pharmacol. Physiol.*. **30**, 96-102.

Asher, N. (1998) Method for detecting a nucleic acid involving the production of a triggering RNA and transcription amplification. *US Patent* No. 5,871,914.

Baner, J., Nilsson, M., Isaksson, A., Mendel-Hartvig, M., Antson, D.-O., Landegren, U. (2001) More keys to padlock probes: mechanisms for high-throughput nucleic acid analysis. *Curr. Opin. Biotechnol.* **12**, 11-15.

Berzal-Herranz, A., Burke, J.M. (1997) Ligation of RNA molecules by the hairpin ribozyme. *Methods Mol. Biol.* **74**, 349-355.

Bohula, E.A, Salisbury, A.J., Sohail, M., Playford, M.P., Riedemann, J., Southern, E.M., Macaulay, V.M. (2003) The efficacy of small interfering RNAs targeted to the type 1 IGF receptor is influenced by secondary structure in the IGF1R transcript. *J. Biol. Chem.*. **278**, 15991-15997.

Bonnet G, Tyagi S, Libchaber A, Kramer FR. (1999) Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proc Natl Acad Sci U S A* **96**, 6171-6.

Braasch, D.A., Corey, D.R. (2002) Novel antisense and peptide nucleic acid strategies for controlling gene expression. *Biochemistry* **41**, 4503-4510.

Brantl S. (2002) Antisense-RNA regulation and RNA interference. *Biochim. Biophys. Acta.* **1575**, 15-25.

Deyev, S.M., Mukhamedov, R.S., Sakharova, N.K., Polyanovsky, O.L., Viklicky, V., Franek, F., Hradec, J. (1984) Purification of mRNA for immunoglobulin kappa-chains from myeloma and hybridoma cells using hybridization to immobilized complementary DNA. *Immunol Lett.* **7**, 315-319.

Dynal (2000) *Biomagnetic Techniques in Molecular Biology. Technical Handbook*, 3rd edition, Dynal AS, Oslo, pp. 87-155.

Esteban, J.A., Banerjee, A.R., Burke, J.M (1997) Kinetic mechanism of the hairpin ribozyme. *J. Biol. Chem.* **272**, 13629-13639.

Fedor, M.J. (2000) Structure and function of the hairpin ribozyme. *J Mol Biol.* **297**, 269-291.

Feldstein, P.A., Bruening, G. (1993) Catalytically active geometry in the reversible circularization of 'mini-monomer' RNAs derived from the complementary strand of tobacco ringspot virus satellite RNA. *Nucleic Acids Res.* **21**, 1991-1998.

Feldstein, PA, Levy, L., Randles, J.W., Owens RA. (1997) Synthesis and two-dimensional electrophoretic analysis of mixed populations of circular and linear RNAs. *Nucleic Acids Res.* **25**, 4850-4854.

George, S.T., Shih, A., Bockman, J.M. (1998) Regulatable nucleic acid therapeutic and methods of use thereof. *US Patent* No. 5,741,679.

Gryaznov, S., Lloyd, D.H. (1998) Oligonucleotide clamps having diagnostic and therapeutic applications. *United States Patent* No. 5,817,795.

Hohjoh, H. (2002) RNA interference (RNAi) induction with various types of synthetic oligonucle duplexes in cultured human cells. *FEBS Lett.* **521**, 195-199.

Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E., Prydz, H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Res.* **30**, 1757-1766.

Jiang, M., Milner, J. (2002) Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* **21**, 6041-6048.

Johnston, B.H., Kazakov, S.A., Alizadeh, B., Kisich, K.O. (2000) RNA Padlocks: A potential new approach to gene inhibition. *Fifth Annual Meeting of the RNA Society.* Poster & Oral Presentation. Abstract book, p. 415. May 30-June 4, Madison, WI.

Johnston, B.H., Kazakov, S.A., Kisich, K. (1998) Antisense and antigene therapeutics with improved binding properties and methods for their use. *International Patent Application (PCT)* WO99/09045A1.

Johnston, B.H., Vlassov, A.V., Alizadeh, B.N., Austin, I., Schweitzer, C., Lundstrom, K., Seyhan, A.A. & Kazakov, S.A. (2002) Libraries of RNA Lassos and ribozymes for cell-based selection of mRNA sequences accessible to antisense agents. *Seventh Annual*

Meeting of the RNA Society. Poster & Oral Presentation. Abstract book, p. 529. May 28-June 2, Madison, WI.

Kazakov, S.A., Vlassov, A.V., Johnston, B.H. (2002) Methods and compositions for production of directed sequence libraries. *U.S. Provisional Application* No. 60/383,208 (filed on May 24, 2002).

Kisich, K.O., Malone, R.W., Feldstein, .A., Erickson, K.L. (1997) Specific inhibition of macrophage TNF-alpha expression by in vivo ribozyme treatment. *J. Immunol.* **163**, 2008-2016.

Kitabwalla, M., Ruprecht, R.M. (2002) RNA interference - a new weapon against HIV and beyond. *N. Engl. J. Med.* **347**, 1364-1367.

Komatsu, Y., Kanzaki, I., Koizumi, M., Ohtsuka, E. (1995) Modification of primary structures of hairpin ribozymes for probing active conformations. *J. Mol. Biol.* **252**, 296-304.

Komatsu, Y., Koizumi, M., Nakamura, H., Ohtsuka, E. (1993) Loop-size variation to probe hairpin ribozymes. *J. Am. Chem. Soc.* **116**, 3692-3696.

Li Q, Luan G, Guo Q, Liang J. (2002) A new class of homogeneous nucleic acid probes based on specific displacement hybridization. *Nucleic Acids Res.* **30**(2), E5.

Lichtenstein , C., Nellen, W., eds. (1998) *Antisense Technology. A practical Approach* (The Practical Approach Series **185**), Oxford University Press, Oxford-New York.

Mercatante, D.R., Bortner, C. D., Cidlowski, J. A., Kole, R. (2001) Modification of Alternative Splicing of Bcl-x Pre-mRNA in Prostate and Breast Cancer Cells. *J. Biol. Chem.* **276**, 16411–16417.

Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdhary, B.P., Landegren, U. (1994) Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science* **265**, 2085-2088.

Noonberg, S.B., Scott, G.K., Garovoy, M.R., Benz, C.C., Hunt, C.A. (1994) In vivo generation of highly abundant sequence-specific oligonucleotides for antisense and triplex gene regulation. *Nucleic Acids Res.* **22**, 2830-2836.

Opalinska, J.B., Gewirtz, A.M. (2002) Nucleic-acid therapeutics: basic principles and recent applications, *Nat. Rev. Drug Discov.* 1, 503-514.

Pan, D., Gambhir, S.S., Toyokuni, T., Iyer, M.R., Acharya, N., Phelps, M.E., Barrio, J.R. (1998) Rapid synthesis of a 5'-fluorinated oligodeoxy-nucleotide: a model antisense probe for use in imaging with positron emission tomography (PET). *Bioorg. Med. Chem. Lett.* **8**, 1317-1320.

Patzel, V., Sczakiel, G. (2000) In vitro selection supports the view of a kinetic control of antisense RNA-mediated inhibition of gene expression in mammalian cells. *Nucleic Acids Res.* **28**, 2462-2466.

Porta, H., Lizardi, P.M. (1995) An allosteric hammerhead ribozyme. *Biotechnology* **13**, 161-164.

Probst, J.C., Skutella, T. (1996) Elevated messenger RNA levels after antisense oligodeoxynucleotide treatment in vitro and in vivo. *Biochem. Biophys. Res. Commun.* **225**, 861-868.

Roberts, R.W., Crothers, D.M. (1992) Stability and properties of double and triple helices: dramatic effects of RNA or DNA backbone composition. *Science* **258**, 1463-1436.

Robertson, M.P., Ellington, A.D. (2000) Design and optimization of effector-activated ribozyme ligases. *Nucleic Acids Res* **28**, 1751-1759.

Rossi, J.J. (1999) Ribozymes, genomics and therapeutics. *Chem. Biol.* **6**, R33-7.

Rupert, P.B, Ferre-D'Amare, A.R. (2001) Crystal structure of a hairpin ribozyme-inhibitor complex with implications for catalysis. *Nature* **410**, 761-763.

Saha, S., Sparks, A.B., Rago, C., Akmaev, V., Wang, C.J., Vogelstein, B., Kinzler, K.W., Velculescu, V.E.

(2002) Using the transcriptome to annotate the genome. *Nat Biotechnol.* **20**, 508-512.

Seyhan, A.A., Alizadeh, B., Austin, I. Kazakov, S.A. Schweitzer, C., Lundstrom, K., Johnston, B.H. (2001) Virally-expressed RNA Padlocks inhibit TNF-α protein synthesis. *Nucleic Acids Res. Symp. Ser.* **42**, 62-63 (*Symposium on RNA Biology IV. RNA: Target and Tool*, October 19-21, Chapel Hill, NC).

Seyhan, A.A., Diegelman, A. M., Kool, E.T., Burke, J. M. (2002) Rolling circle transcription of ribozymes from promoterless single-stranded DNA nanocircles in mammalian cells, *submitted* to RNA.

Shi, Y. (2003) Mammalian RNAi for the masses. *Trends Genet.* **19**, 9-12.

Shih, A., Bockman, J.M., George, S.T. (1996) Ribozyme amplified diagnostics. *US Patent* No. 5,589,332.

Sohail, M., Southern, E.M. (2000) Selecting optimal antisense reagents. *Adv. Drug Deliv. Rev.* **44**, 23-34.

Stein, C.A., Krieg, A.M., eds. (1998) *Applied Antisense Oligonucleotide Technology.* Willey-Liss, New York-Toronto.

Stein, C.A. (2000) Is irrelevant cleavage the price of antisense efficacy? *Pharmacol. Ther.* **85**, 231-236.

Stiege, W., Kosack, M., Stade, K., Brimacombe, R. (1988) Intra-RNA cross-linking in Escherichia coli 30S ribosomal subunits: selective isolation of cross-linked products by hybridization to specific cDNA fragments. *Nucleic Acids Res.* **16**, 4315-4329.

Sullenger, B.A., Gilboa, E. (2002) Emerging clinical applications of RNA. *Nature.* **418**, 252-258.

Tavitian, B., Terrazzino, S., Kuhnast, B., Marzabal, S., Stettler, O., Dolle, F., Deverre, J.R., Jobert, A., Hinnen, F., Bendriem, B., Crouzel, C., Di Giamberardino, L. (1998) *In vivo* imaging of oligonucleotides with positron emission tomography. *Nat. Med.* **4**, 467-471.

Taylor, J.K., Zhang, Q.Q., Wyatt, J.R., Dean, N.M. (1999) Induction of endogenous Bcl-xS through the control of Bcl-x pre-mRNA splicing by antisense oligonucleotides. *Nat Biotechnol.* **17**, 1097-1100.

Toulme, J.-J. (2001) New candidates for true antisense. *Nat. Biotechnol.* **19**, 17-18.

Tuschl, T. (2002) Expanding small RNA interference. *Nature Biotechnol.* **20**, 446-448.

Tyagi, S., Kramer, F.R., Lizardi, P.M. (1998) Sensitive nucleic acid sandwich hybridization assay. *US Patent No.* 5,759,773.

Vickers T.A., Koo, S., Bennett, C.F., Crooke, S.T., Dean, N.M, Baker, B.F. (2003) Efficient Reduction of Target RNAs by Small Interfering RNA and RNase H-dependent Antisense Agents. A COMPARATIVE ANALYSIS. *J. Biol. Chem.* **278**, 7108-17108.

Walton, S., Stephanopoulos, G.N., Yarmush, M.L., Roth, C.M. (1999) Prediction of antisense oligonucleotide binding affinity to a structured RNA target. *Biotechnol. Bioeng.* **65**, 1-9.

Zamore, P.D. (2002) Ancient Pathways Programmed by Small RNAs. *Science* **296**, 1265-1269.

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[ ] *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

METHOD OF PREPARATION OF RNA CAPABLE OF TARGET-DEPENDENT CIRCULARIZATION AND TOPOLOGICAL LINKAGE

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ENCLOSED APPLICATION PARTS (*check all that apply*)

[x] Specification *Number of Pages* 31 [ ] CD(s), Number [ ]

[x] Drawing(s) *Number of Sheets* 19 [x] Other: Return Postcard

[x] Application Data Sheet. See 37 CFR 1.76 (3 pages)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[x] Applicant claims small entity status. See 37 CFR 1.27.

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[x] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Date June 25, 2003

SIGNATURE *Jill A. Jacobson*

TYPED OR PRINTED NAME Jill A. Jacobson

REGISTRATION NO. *(if appropriate)* 40,030

TELEPHONE (650) 813-5876

Docket Number: 367593000500

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PA-799920

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